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CANCER RESEARCH

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CANCER RESEARCH

VOLUME 9 JANUARY 1949 NUMBER 1

Announcement

IT IS a pleasure to announce that with this issue *CANCER RESEARCH* is appearing under the auspices of a new Editorial Board and with a new Publisher. Owing to the war and subsequent difficulties of nearly all kinds, there have been great delays in the publication of this journal which we hope have now ended.

CANCER RESEARCH is the official organ of publication of the AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC. At the annual meeting of the Association in Atlantic City in 1948, the Board of Directors authorized the formation of a holding company for *CANCER RESEARCH*, the officers of which were to be identical with the officers of the Association. This has been established, and the journal is now owned and published by the new corporation, CANCER RESEARCH, INC. The Board of Directors of the Association have considered it essential to fulfil two obligations:

1. To give prompt and wide dissemination of important research in the field of cancer;
2. To attempt to reduce the financial deficit under which this publication, *CANCER RESEARCH*, has been operating.

The officers of CANCER RESEARCH, INC., as well as the new and distinguished Board of Editors and the Publisher, will make every effort to accomplish these ends. They bespeak the same generous and enthusiastic co-operation of the members of the AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC., our sponsors, and of the research workers in problems of neoplastic growth that *CANCER RESEARCH* has enjoyed in the past.

CHARLES HUGGINS, *President*

Editorial Foreword

WITH the advent of a new editorial staff, it is customary to make a statement regarding editorial policy. *CANCER RESEARCH* is fortunate in that first its Editorial Committee and later its editors, Dr. William H. Woglom, Dr. S. Bayne-Jones, and Dr. Balduin Lucké, with their staffs, have set high standards. It is our present policy to emulate them, but the general editorial policy will remain unchanged. Dr. Lucké, who has had to relinquish the position of editor-in-chief because of press of other duties, has generously consented to remain as an editor, giving to the new editorial staff the benefits of his experience.

CANCER RESEARCH is devoted to reporting the results of fundamental investigations directed toward the understanding and ultimate conquest of cancer. Original reports of laboratory and clinical research, chiefly experimental, will be published regardless of whether the subjects are mice or men. Comprehensive but concise general reviews of subjects selected by the editorial staff will be published from time to time. Short papers, even preliminary, if so marked, will be welcome. News items, announcements, comments, and death notices will also be published. Other sections will be added as warranted. It is hoped that such prompt publication can be offered to papers of wide and active interest that a copy of *CANCER RESEARCH* will be a necessity on the desk of every scientist and physician concerned with cancer.

With the great expansion in cancer research now under way and the limitation of printing space dictated by budgetary considerations, it will be impossible to print all papers submitted to the editors. Critical selection beween them will be necessary, and some authors will, of necessity, be disappointed. Past policy of careful editorial selection by specialists in each field will be rigidly followed. The role of the editors and advisory editors will be to help in the selections, but the final responsibility must rest with the editor-in-chief, who alone will have access to all editorial opinions and be able to judge the apparent comparative merit of the papers submitted. Knowing that, regardless of how carefully the selections are made, some persons will disagree with the choices, the editor-in-chief is fully reconciled to receiving adverse criticism and to loss of all of his friends if this becomes necessary in order to maintain what he regards as the highest standards for *CANCER RESEARCH*. It is expected that *CANCER RESEARCH* can eventually be enlarged.

It is hoped that in these pages, among other benefits, investigators will find an adequate and prompt publication outlet for their results, readers a fertile and reliable source of information, physicians a flood of ideas and inspiration which they will use in the clinic, and the sponsors satisfaction in having had a vital part in an important job well done.

PAUL E. STEINER

A Comparison of Activators of Proteolytic Enzymes and Peptidases in Normal Rat Livers and Hepatomas*

PAUL C. ZAMECNIK, M.D., AND MARY L. STEPHENSON

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital, of Harvard University, located at the Massachusetts General Hospital, Boston 14, Massachusetts)

The more rapid growth rate of a malignant tissue as compared with its normal counterpart implies either an increased rate of protein synthesis or a decreased rate of protein degradation in the malignant cells. Although the work of Schoenheimer (26) indicates that protein is in a constant state of turnover in the living animal, there is scant information available on the nature of the forces which control protein synthesis and degradation.

The studies of Bergmann, Fruton, and their coworkers (2, 3) make it appear likely that catheptic enzymes play an important role in the process of proteolysis within the cell. This family of enzymes operates at a weakly acid pH and (with a single exception) requires the presence of certain activating substances such as glutathione or cysteine in order to function. It thus appears reasonable to look for the presence of such activators of proteolysis within normal and malignant liver tissues.

A recently described manometric method (11, 30) has made it possible to study the activation and kinetics of certain catheptic enzymes with greater facility than has hitherto been possible.

EXPERIMENTAL

Animals.—Seventy young male Harvard colony[1] rats were divided into three groups and were placed on the following diets: (*a*) *p*-dimethylaminoazobenzene diet of Kline and his associates (21) (Table 1, group 4); (*b*) same as (*a*), but without *p*-dimethylaminoazobenzene; (*c*) normal control diet (Pratt's Nurishmix[2]). At the end of 4 months, groups 1 and 2 were transferred from the experimental diet to control diet *c*. Between the seventh and ninth months, rats were taken at random from each group and were killed by exsanguination. Only animals bearing grossly visible hepatomas were used in the study. Sections of livers were saved in all cases for histological examination. The hepatoma nodules were principally parenchymal cell in type, corresponding to hepatoma type II of Edwards and White (9). In a number of tumors there were also smaller areas consisting of adenocarcinoma, hepatoma type I, and bile duct proliferation. Peritoneal metastases were frequently present. The remainder of these livers

TABLE 1

EFFECT OF ULTRAFILTRATES ON THE ACTIVITY OF A DIALYZED CATHEPTIC ENZYME, USING CARBOBENZOXYGLYCYL-*l*-TYROSINE AS SUBSTRATE*

No ultrafiltrate	Control ultrafiltrate	Hepatoma ultrafiltrate	Ultrafiltrate from nonmalignant part of hepatoma-containing liver
6.7	39.7	14.7	64.4
6.0	33.6	8.7	29.6
6.7	32.3	4.0	45.6
4.4	30.2	4.7	43.1
5.1	38.3	4.7	49.2
	47.0	37.6	39.0
	46.5	13.4	56.3
	37.3	25.5	23.5
	38.4	7.4	61.2
		25.9	34.3
		4.7	57.6
		34.3	61.6
		5.7	32.5
		22.5	
		12.1	
		5.7	
5.8	37.0†	14.5	46.0†

* The figures signify $10^9 \times$ moles of carbon dioxide liberated per cubic centimeter of test solution per minute. One hundred per cent hydrolysis of the substrate would represent 39×10^{-6} moles of CO_2 liberated per cubic centimeter of test solution. Each figure represents a determination on a separate rat. Since the same enzyme and substrate have been used throughout, the differences shown above represent differences in enzyme activator concentration present in the system due to addition of ultrafiltrate.

† Difference from hepatoma ultrafiltrate significant ($p < 0.01$) (1).

* Supported by the Godfrey M. Hyams Fund and by a grant-in-aid from the American Cancer Society (recommended by the Committee on Growth of the National Research Council).

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[1] Rats obtained originally from Sprague-Dawley and inbred periodically in Hisaw's laboratory by brother- and sister-matings during the last 15 years.

[2] Stated ingredients as follows: dried buttermilk, beef scrap, wheat-germ meal, rolled oats, molasses, iodized salt, cod-liver oil.

usually showed evidence of diffuse, nodular cirrhosis of a mild degree but was grossly nonmalignant. Thus in a single animal there were present both malignant and nonmalignant areas of liver, clearly separated from each other (Fig. 1). We have found that, under similar conditions, rats left on Kline's diet (21) for 6 months or more develop livers studded and infiltrated with hepatomas, with little tissue remaining that can be termed nonmalignant or normal. On the other hand, if the dye is discontinued at the end of the fourth month, a relatively few hepatomas arise in isolated areas of a single liver. We have, therefore,

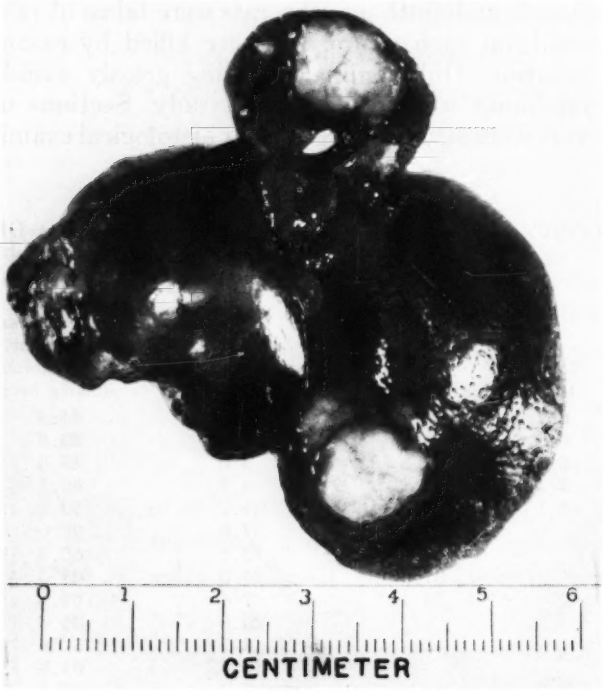


FIG. 1.—Typical liver, showing hepatoma nodules

taken advantage of this circumstance in preparing tumors for the present experiment.

The opaque, white hepatoma nodules were carefully dissected free from surrounding grossly nonmalignant tissue. Hepatoma nodules obtained from a single liver varied in size from a few millimeters to 1 to 1.5 cm. In the case of the larger nodules, particular care was taken to select only the peripheral, grossly non-necrotic, firm portions. Sections were also taken from such large nodules for histological verification. Thus a quantity of hepatoma nodules weighing from 3 to 10 gm., usually the former, was obtained from a single liver. In a number of experiments an equal weight of tissue was likewise taken from the grossly nonmalignant parts of these livers, and histological sections were saved from these regions. At the same time, a control liver was frequently obtained and prepared as described above.

Ultrafiltration.—A 1:1 homogenate of liver or hepatoma was quickly made with distilled water, using a Potter-Elvehjem apparatus (24). The homogenized livers were then placed individually in small Cellophane bags[3] inside side-armed Erlenmeyer flasks in the cold-room at 3° C. and were allowed to filter under the influence of a negative

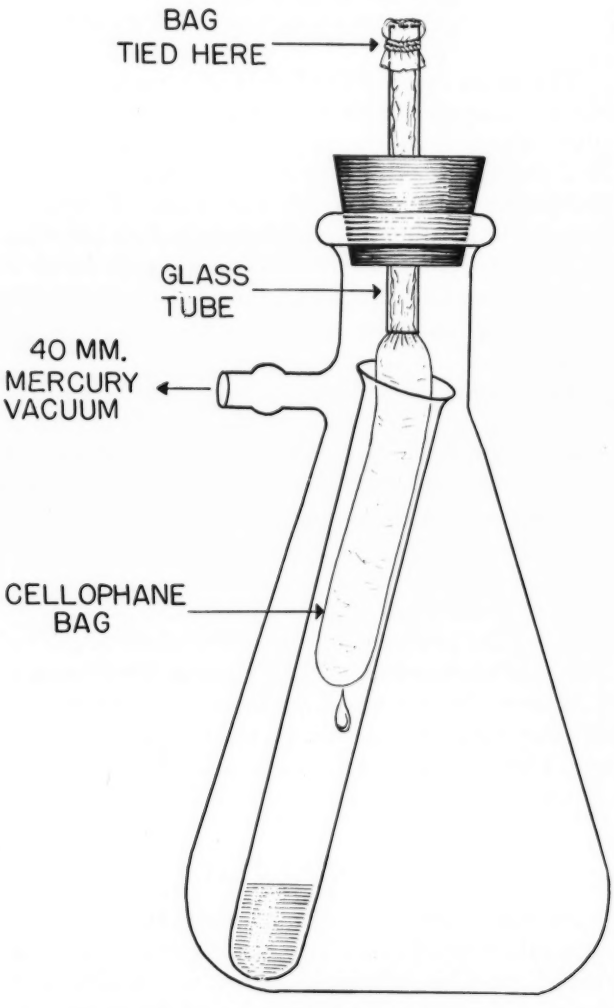


FIG. 2.—Ultrafiltration apparatus

pressure of approximately 40 mm. of mercury (Fig. 2). This filtration pressure was selected so as to be just under the breaking-point of the bags. Ultrafiltration was chosen as a method for separating the substances of large molecular weight present in liver tissue from compounds of smaller molecular weight because it seemed to introduce less possibility of artifact than did acid precipitation methods. One to 3 cc. of water-clear, protein-

[3] Visking Cellulose Sausage Casings, 14/32-inch "No Jax" Casing.

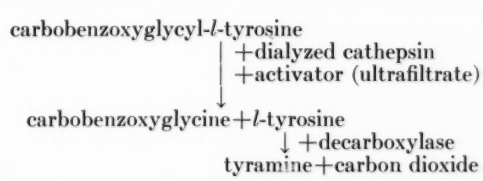
free ultrafiltrate were collected during the following 4 to 6 hours. It was found that, if the ultrafiltrate was allowed to collect overnight, the activating ability of an aliquot was less potent than that of one obtained during the early hours. Storage of ultrafiltrates over a period of days on dry ice, however, resulted in no appreciable loss in their ability to activate the catheptic enzyme.

ENZYMATIC DETAILS

1. *Preparation of cathepsin.*—Several separate preparations of cathepsins were made by ammonium sulfate fractionation of fresh hog kidneys, according to the procedure of Fruton and Bergmann (12). In order to remove, as far as possible, all traces of natural activators, the final products were dialyzed at 3° C. for 9 to 13 days, first against several changes of copper-distilled water and, finally, against glass-distilled water.

The activity of the cathepsin preparations was tested toward the substrate carbobenzoxyglycyl-*l*-tyrosine, by means of a manometric method (30), both in the presence and in the absence of added activator. Each preparation of cathepsin was divided into 10-cc. portions. These were frozen in lustroid tubes in a dry-ice box, were thawed out individually, and used immediately. The frozen enzyme solution retained its activity, but the activity decayed over a period of hours after thawing.

2. *Determination of catheptic carboxypeptidase.*—Although such a preparation as that mentioned above contains a number of catheptic enzymes, it was convenient to study the effect of activators in detail on only two enzymes—the carboxypeptidase which hydrolyzes the substrate carbobenzoxyglycyl-*l*-tyrosine, and the trypsinase, or more strictly benzoylargininamidase (17), which hydrolyzes benzoyl-*l*-argininamide. The catheptic carboxypeptidase method depends on the ability of a tyrosine decarboxylase (obtained from *Streptococcus faecalis*) to liberate carbon dioxide from free *l*-tyrosine, but not from the peptide carbobenzoxyglycyl-*l*-tyrosine. The reaction sequence involved is outlined below:



Reactions were carried out in Warburg-type constant-volume respirometers, at 25° C. The gas phase was nitrogen, purified of traces of oxygen by passage over hot copper. In agreement with previous work (19), we have found higher catheptic activity when the reactions have been run in the absence of oxygen. Unless otherwise stated, the composition of the test solution was as follows:

Five-tenths cubic centimeters of dialyzed cathepsin, 0.3 cc. of 0.055 *M* carbobenzoxyglycyl-*l*-tyrosine,[4] and 0.6 cc. of decarboxylase made up in 0.4 *M* citrate buffer. The pH was 5.4 to 5.6 in various experiments, but in a single experiment there was less than 0.1 pH units difference among the various flasks. The decarboxylase was prepared as previously described (30).

Provided that the decarboxylase is present in excess, the rate of carbon dioxide evolution serves as a measure of the rate of the cathepsin reaction (30). During the early portion of the hydrolysis, the reaction closely follows zero-order kinetics. Likewise, if the dialyzed cathepsin is completely inactive in the absence of added activator, the rate of carbon dioxide evolution reflects the amount of cathepsin activation provided by the ultrafiltrate. Even in the absence of added activator, however, the dialyzed enzyme was able to split this substrate slightly. This suggests that all the natural activator may not have been dissociated from the enzyme during the prolonged dialysis. The relationship between concentration of glutathione added to the test solution and rate of peptide hydrolysis (as reflected by carbon dioxide evolution) is illustrated in Fig. 3.

It may be seen from Fig. 3 that the activity of the dialyzed cathepsin toward the substrate carbobenzoxyglycyl-*l*-tyrosine is a function of the glutathione concentration added to the test solution.

3. *Determination of catheptic trypsinase (benzoyl-argininamidase).*—A smaller number of experiments was performed, in which the action of dialyzed cathepsin preparation on the substrate benzoyl-*l*-argininamide[5] was tested at pH 5 both in the presence and in the absence of added activator. The experimental details were as follows: Reactions were run in 2.5-cc. glass-stoppered volumetric flasks, incubated in a water bath at 37° C in the presence of air. The flasks contained 1.0 cc. of 0.1 *M* benzoyl-*l*-argininamide, 0.2 cc. of 1 *M* citrate buffer at pH 4.72, 0.4 cc. of cathepsin, and 0.4 cc. of ultrafiltrate. With each experiment one control was run in which 0.4 cc. of distilled water was used in place of ultrafiltrate, and another control in which 0.4 cc. of 0.05 *M* cysteine was simi-

[4] The authors are indebted to Dr. Max Brenner and the A. G. Geigy Co., Basle, Switzerland, for generous supplies of carbobenzoxyglycyl-*l*-tryosine and of *l*-leucinamide.

[5] Obtained through the courtesy of Dr. M. S. Dunn and the Amino Acid Manufactures, Los Angeles, California.

larly used. Autolysis controls were also included in which water was used in place of the substrate. There was no autolysis at the end of an hour. Duplicate aliquots were removed at zero time, and the reactions were all terminated at 60 minutes.

The pH's of aliquots from all flasks were checked at the beginning and end of each experiment by means of a micro glass electrode assembly. Aliquots of 0.2 cc. were diluted to 1.2 cc. with distilled water and were tested. With rare exceptions, the pH's at this dilution were between 5.40 and 5.50 throughout the experiments.

The flasks were shaken briefly by inversion at 15-minute intervals during the course of the reaction. Individual flasks were removed from the warm bath at 60 minutes, placed in ice water, and three 0.2-cc. aliquots were removed. The aliquots were pipetted into aeration tubes containing 5 drops of caprylic alcohol, 2 drops of castor oil,

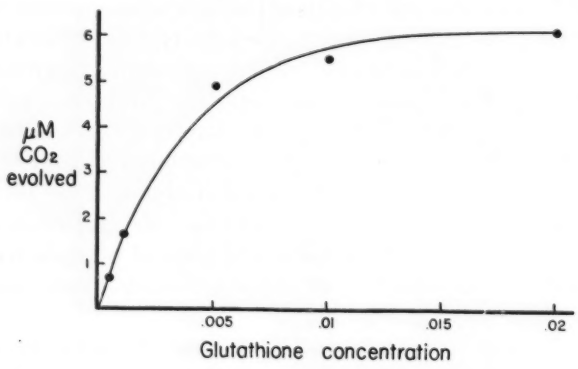


FIG. 3.—Relationship of cathepsin activity to glutathione concentration. The flask contents were as follows: 0.5 cc. of 13-day dialyzed cathepsin; 0.3 cc. of 0.055 *M* carbobenzoxyglycyl-*l*-tyrosine; 0.6 cc. of decarboxylase (20 mg. of dried powder per cubic centimeter of 0.4 *M* citrate buffer, pH 5.3); and 0.5 cc. of glutathione solution. The pH's of the various flasks, as determined at the end of 1 hour's reaction, ranged from 5.25 to 5.43. The temperature was 27° C., and the gas phase was purified nitrogen. A control flask was run in the absence of glutathione, and the value obtained (0.7 μ moles of CO_2 liberated per hour) was subtracted from the other readings. Glutathione concentration is expressed in moles per liter, and carbon dioxide in μ moles evolved per hour.

0.5 cc. of a half-saturated solution of potassium carbonate, and 0.8 cc. of distilled water. The tubes were stoppered, and aeration was begun immediately. The ammonia was collected in boric acid and was titrated (27), using 0.0782 *N* sulfuric acid and a Rehburg burette. The average of the replicate titrations was used in each case. In following the splitting-off of the amide group by the cathepsin, ammonia aeration, according to a modification of the method of Sobel, Meyer, and Gottfried (27), was found to be more convenient and accurate in our hands than was the more classical titration procedure of Grassmann and Heyde (15).

More effort was expended on studies on carbobenzoxyglycyl-*l*-tyrosine, however, than on those with benzoyl-*l*-argininamide, because of the greater precision and ease of manipulation of the manometric method used in the study of the enzymatic hydrolysis of the former substrate.

4. *Determination of* l-*leucinamidase.*—This enzyme was used to provide an example of the behavior of the group of metal-activated peptidases whose pH optimum is in the vicinity of pH 8. A crude preparation of peptidase was made from rat liver in the following way: 9 gm. of normal rat liver were minced and then homogenized with 27 cc. of water. The homogenate was twice filtered through a Buchner funnel with the aid of Hy-Flo Supercel. To the filtrate was added an equal volume of ice-cold acetone. The precipitate was washed 3 times with ice-cold acetone. To 300 mg. of dried precipitate, 18 cc. of water were added. The extract was centrifuged, and 17 cc. of a clear, reddish supernatant solution were obtained. This solution contained an enzyme which actively hydrolyzed *l*-leucinamide at pH 8.

The experimental details were as follows: flasks contained 1.0 cc. of 0.1 *M l*-leucinamide (previously adjusted to pH 8); 0.2 cc. of 0.4 *M* borate buffer at pH 8; 0.4 cc. of a 1.5 aqueous dilution of enzyme solution; 0.1 cc. of 0.02 *M* manganese chloride (when used); 0.4 cc. of ultrafiltrate (when used); and distilled water to 2.0 cc. The reaction temperature was 37° C. At 15-, 30-, and 45-minute intervals, 0.2-cc. aliquots were removed and were titrated according to the technic of Grassmann and Heyde (15).

5. *Determination of reduced glutathione.*—The most specific procedure for the determination of tissue glutathione concentration is the manometric method of Woodward (29). This method depends on the ability of reduced glutathione to serve as a coenzyme for the yeast enzyme, glyoxalase, in catalyzing the conversion of methylglyoxal to lactic acid. The lactic acid formed during the reaction displaces carbon dioxide from a carbon dioxide–bicarbonate buffered system, and the carbon dioxide evolution is measured manometrically. Several batches of yeast were tested for glyoxalase activity, and calibration curves were set up, in which varying amounts of glutathione were added to the glyoxalase. It was found impossible to free the yeast glyoxalase completely from its naturally accompanying coenzyme. Even without the addition of glutathione, therefore, there was considerable glyoxalase activity. Results have been corrected for this blank value in each experiment by running flasks without added glutathione.

RESULTS

A. Activating Effect of Ultrafiltrates on Dialyzed Cathepsin

1. *Results on catheptic carboxypeptidase.*—In Table 1 is tabulated the effect of ultrafiltrates on the activity of dialyzed cathepsin, using the substrate carbobenzoxyglycyl-*l*-tyrosine. It will be noted that there is some enzymatic activity present even when no ultrafiltrate is added to activate the cathepsin. Apparently, not all the natural cathepsin activator is removed by dialysis. The average of the values in the first column of Table 1 may thus be subtracted from the averages of values in the other columns to obtain figures for the amount of activation provided by the addition of the three types of ultrafiltrates. *The results indicate less activating ability in the ultrafiltrates of hepatoma nodules than in the ultrafiltrates from the nonmalignant parts of the same livers.* There is likewise less activating ability in the hepatoma ultrafiltrates of livers of control rats.

TABLE 2

Effect of Ultrafiltrates on the Activity of a Dialyzed Catheptic Enzyme Using Carbobenzoxyglycyl-*l*-Tyrosine as Substrate*

No ultrafiltrate	Control ultrafiltrate	Regenerating liver	Fetal liver	Hepatoma
6.7	17.1	28.0	46.8	8.3
5.0	26.0	26.9	26.6	17.5
6.5	26.6	10.6	33.2	14.4
	17.3	29.2	45.5	7.0
	16.1	29.2	46.8	8.1
	20.6	18.6		12.1
	17.4			25.0
	26.4			
6.1	21.0†	23.7†	39.8‡	13.4

* Experimental details same as those given in Table 1.
† Difference from hepatoma ultrafiltrate probably significant ($p < 0.05$).
‡ Difference from hepatoma ultrafiltrate significant ($p < 0.01$).

In Table 2 are listed the results of a completely separate series of experiments in which two additional types of control livers were used, namely, regenerating and fetal livers. The regenerating livers were produced by performing partial hepatectomy (5), with removal of the regenerating liver 24 hours later. Each figure in the fetal liver column represents an ultrafiltrate obtained from the pooled livers of the fetuses of a single mother. As before, hepatoma nodules were carefully dissected out of individual livers. Another preparation of cathepsin was used for these experiments. Since the order of activity of this enzyme preparation, per milligram of protein nitrogen, was considerably less than that of the previous preparation; and, because the amount of natural activator dialyzed out might also be different, this series of experiments must be viewed as a unit separate from those in Table 1.

The results again show a lower activation effect of the hepatoma nodule ultrafiltrates than that found in the controls. It is noteworthy that the activating ability of the fetal ultrafiltrates is particularly high.

2. *Results on benzoylargininamidase (trypsinase).*—The effect of ultrafiltrates on benzoylargininamidase activity is summarized in Table 3. It will be noted that the dialyzed enzyme has little activity in the absence of added activator. The addition of 0.01 *M* cysteine or glutathione provides maximal activation. Although there is overlapping of values in the two series, the average concentration of benzoylargininamidase activator is less in the hepatoma than in the control ultrafiltrates. The results thus fall in line with those on the activation of catheptic carboxypeptidase.

TABLE 3

Effect of Ultrafiltrates on Benzoylargininamidase Activity*

(1) No activator added	(2) 0.01 M Cysteine added	(3) Control ultrafiltrate added	(4) Hepatoma ultrafiltrate added
1.9	62	9.8	2.6
1.2	59	9.9	9.6
2.7	55	9.4	3.4
2.0	62	5.6	1.6
1.6	65	6.3	4.8
1.3	61	9.3	10.8
2.0	58	9.7	8.5
1.6	58	10.4	2.1
1.5	59	7.8	1.5
0.4†	40	7.9	1.2 2.0
1.3†	42	8.8 10.1	5.1
1.4†	45	6.8 5.2	5.7
1.6	55	8.4	4.5‡

* Each horizontal line represents an experiment carried out on a single day. Each figure in cols. 3 and 4 represents a separate liver. Results are expressed as $10^2 \times$ moles split per hour per 0.2 cc.-aliquot titrated. One hundred per cent hydrolysis of substrate on this basis would represent 100×10^{-7} moles.

† A second preparation of enzyme was used for the last 3 experiments. The maximal activity per milligram protein nitrogen was somewhat lower than that of the previous preparation. A correction could conceivably be applied in order to bring the whole series of figures for the last 3 experiments more closely in line with the previous ones. This has not, however, been done.

‡ Difference from control ultrafiltrate significant ($p < 0.01$) (1).

3. *Results on l-leucinamidase.*—The enzyme used in these few experiments was prepared from rat liver as described above. When manganese chloride was added to the test solution, the enzyme hydrolyzed *l*-leucinamide very actively (50 per cent of the substrate was hydrolyzed in 45 minutes). Without the addition of manganese, however, there was only slight activity. The addition of ultrafiltrates of a normal liver and of a hepatoma produced no activating effect. This en-

zyme is, of course, quite different from the cathepsins discussed above. The results thus indicate that no appreciable amount of activator for this enzyme has been found in ultrafiltrates of either normal or malignant liver by this technic.

B. Studies on Chemical Nature of Cathepsin Activators Present in Ultrafiltrates

1. *Nitroprusside test.*—Since it has been known (13) that certain compounds containing sulfhydryl groups may serve as cathepsin activators, frozen aliquots of the ultrafiltrates were thawed and tested for their total sulfhydryl activity by means of the nitroprusside reaction (18). The colorimetric results could be roughly quantitated and are summarized in Table 4. The figures suggest that the sulfhydryl concentration of the hepatoma is less than that in the normal livers, in regenerating liver, and in the nonmalignant part of the hepatoma-containing livers. The sulfhydryl concentration of the fetal livers was, however, only slightly higher than that of the hepatomas. It thus appeared desirable to characterize more closely the nature of the sulfhydryl-containing compounds responsible for the nitroprusside test.

TABLE 4

Sodium Nitroprusside Test of Sulfhydryl Group Concentrations in Ultrafiltrates of Various Types of Rat Livers*

Type of liver				
Control	Hepatoma	Control-hepatoma†	Fetal	Regenerating
60	0–5	60	20	100
60	5	80	15	60
50	0–5		5	80
60	0–5		5	
60	0–5		15	
80	0–5			
60				

* Each figure represents a determination on a single liver. An arbitrary color standard was set at 100, and the figures have meaning only in relation to one another.

† This term signifies the grossly nonmalignant portion of a liver containing a hepatoma.

2. *Filter-paper chromatograms.*—Such chromatograms of ultrafiltrates of normal livers, prepared according to Consden, Gordon, and Martin (7), indicated the presence of glutathione in considerable concentrations and a relative paucity of cysteine.

3. *Glyoxalase method.*—The glyoxalase method of Woodward (29)[6] was then used to quantitate the concentration of reduced glutathione. The results of these studies are recorded in Table 5.

The points of interest are the low glutathione concentrations of the hepatoma nodules and of the fetal livers. Thus the low cathepsin-activating ability of the hepatomas appears to fit well with their low, reduced glutathione concentrations. This explanation cannot, however, hold when applied to the fetal liver ultrafiltrates. In the latter case there is a high cathepsin-activating ability but a low reduced glutathione concentration.

The possibility was considered that the glutathione might be present in oxidized form in the hepatoma and thus not be detectable by the glyoxalase method, which measures only reduced glutathione. To test this possibility, the electrolytic reduction method of Dohan and Woodward (8) was set up. By this method glutathione in the oxidized form is reduced and then tested as before by the glyoxalase method. It was found that very little oxidized glutathione was present in the hepatomas tested, and that this, therefore, could not be the explanation for the low concentrations of glutathione found in the hepatomas.

TABLE 5

Glutathione Concentration of Various Types of Ultrafiltrates*

Control liver	Regenerating liver	Fetal liver	Control-hepatoma†	Hepatoma
0.093	0.117	0.013	0.079	0.018
.078	.108	.020	.112	.000
.116	.058	.018	.071	.005
.125	0.050	.033	.093	.003
.091		0.018	.117	.011
.080			0.068	.009
.071				.009
.118				.038
0.085				.013
				.020
				.009
				.003
				.000
				.006
				0.003
0.095	0.083	0.020	0.090	0.010

* Figures indicate milligrams glutathione per 0.1 cc. of ultrafiltrate.

† Nonmalignant part of hepatoma-containing liver.

4. *Sulfosalicylic acid filtrates.*—In order to check on the possibility that ultrafiltration through the Cellophane bag might introduce some unexpected artifact with respect to the glutathione concentrations of the various groups of livers, a series of sulfosalicylic acid filtrates of control livers and hepatomas were run in parallel on the same livers from which the ultrafiltrates were prepared. The glutathione concentrations were in satisfactory agreement by use of the two independent methods.

DISCUSSION

In order to bring the result into focus with current conceptions (26, 25) of protein metabolism within a living cell the following diagrams are pre-

[6] The dihydroxyacetone from which methylglyoxal was prepared was kindly furnished by Drs. Fritz Lipmann and C. B. Anfinsen.

sented (cf. Fig. 4). In a normal adult cell (*N*) in nitrogen equilibrium (*1.*), protein is being synthesized at a rate (*A*) similar to that (*B*) at which it is being degraded. There is thus no net change in the intracellular protein concentration. In a malignant cell (*M*), protein may either be synthesized at an accelerated rate (*2.*), degraded at a decreased rate (*3.*), or both aspects of the process may be affected (*4.*).

There are at least 3 families of enzymes which appear to be concerned in protein and peptide degradation within cells: (*a*) the cathepsins, active at weakly acid pH's; (*b*) the peptidases, active at neutral and slightly alkaline pH's; and (*c*) the more obscure dehydropeptidases. The present experiments suggest that the concentration of cathepsin activators may be decreased in the hepatomas. Thus the present results are consonant with either situation (*3.*) or (*4.*).

We have recently (32) found C^{14}-labeled alanine to be incorporated into the proteins of primary rat hepatoma slices more rapidly than into the proteins of normal liver slices. These data imply an increase in the rate of protein synthesis in the hepatoma *in vitro* but give no information on the rate of protein degradation. In summary, these two fragmentary pieces of evidence, therefore, suggest that situation (*4.*) (Fig. 4) may be the correct one for this type of hepatoma. It is, of course, possible that either situation (*2.*) or situation (*3.*) (Fig. 4) may apply for other tumors.

The question as to whether the catheptic enzymes play a role in protein *synthesis* within the cell, as well as in protein degradation, has, of course, not been settled. Recent work from several laboratories (4, 6, 10, 23), however, favors the point of view that a different set of enzymes is involved in protein synthesis from those concerned with protein degradation.

The data on fetal livers require special comment. Since the glutathione concentration is low but the cathepsin activator concentration high in the ultrafiltrates of fetal liver, there must be some substance other than glutathione responsible for this high order of activation. The nature of such a hypothetical substance is unknown.

It has been pointed out recently (22) that the glutathione concentration of rat livers depends on the diet fed. In the present experiments, animals in all groups except fetal had been on the same control diet for 2 months, prior to sacrificing, which minimizes the possibility that variations in diet might have affected the glutathione concentrations of the several groups.

The glutathione concentration of normal and malignant tissues has been the subject of considerable study, as pointed out in a recent review (28). Fewer comparisons, however, have been made between glutathione concentrations of malignant tissues and their homologous normal counterparts (14, 16, 20). The present findings are in qualitative agreement with those of Kinosita (20) and Greenstein (16). The glutathione concentration in the hepatomas in the present experiments is, however, lower than that found by these investigators. This point, therefore, requires a word of explanation. The experimental details differed in several important respects in the three investigations: (*a*) strain of rat; (*b*) use of primary hepatoma nodules in the present experiments as against transplanted hepatomas in Greenstein's experiments; (*c*) use of the more specific glyoxalase method for determining glutathione in the present experiments; and (*d*) possibility of a greater amount of necrosis in the hepatomas used in the

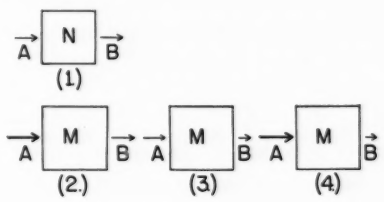


FIG. 4.—Protein metabolism in normal and malignant cells

present experiment. In the necrotic portions of tumors the sulfhydryl concentration is reported (16) to drop nearly to zero. We have been careful, therefore, to choose only the peripheral, grossly non-necrotic portions of the hepatoma nodules for study. Pieces from each hepatoma were saved for histological study. While there was microscopic evidence of some degree of necrosis present in nearly every hepatoma, necrosis was inconspicuous in all but a few sections studied. This latter seems, therefore, to be an unlikely explanation of the glutathione differences.

The primary hepatoma was used, since it affords the advantage of providing both malignant and control tissue of the same genetic origin, growing under similar environmental circumstances.

In a previous paper (31) the activity of catheptic enzymes in 30 per cent glycerol extracts of *p*-dimethylaminoazobenzene-induced primary hepatomas was studied. In such experiments cysteine was invariably added to the test solutions in order to provide maximal activation of the enzymes. Thus the experiments were designed to uncover possible difference in the concentration of the protein part of the enzymes in the hepatomas and control livers. Under these conditions the activity of catheptic trypsinase (benzoylar-

gininamidase) was found to be increased in the hepatoma. In the present experiments the focus has been directed toward the other side of this problem, namely, the concentration of cathepsin activators in hepatomas as compared with control livers. Thus in the hepatoma there is an increased concentration of the protein part of a catheptic enzyme but a decreased concentration of the activator of that same enzyme.

SUMMARY

The cathepsin-activating ability of ultrafiltrates prepared from hepatomas has been found to be considerably less than that of various types of control livers. Since sulfhydryl groups are the most common cathepsin activators found in tissues, we have looked for a possible correlation of the above finding with the glutathione concentrations of the hepatomas. The hepatoma ultrafiltrates had both the lowest glutathione concentrations and the least cathepsin-activating power. The fetal livers, however, had a low glutathione concentration but the greatest cathepsin-activating ability. It is evident, therefore, that the cathepsin may be activated by more than one tissue component.

Glutathione appears (in the normal liver, in the regenerating liver, and in the hepatoma) to be a major cathepsin-activating agent and to be particularly low in the hepatoma. It is thus possible that the increased growth rate of this type of hepatoma may be related to a decrease in the concentration of the glutathione component of one of its protein-degrading mechanisms.

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A Chemical Investigation of Keratin and Carcinomas Deriving from Rabbit Papillomas (Shope)*

HENRY W. SCHERP, PH.D., AND JEROME T. SYVERTON, M.D.†

(From the Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester 7, New York)

In 1933, Shope (14) described a virus-induced papilloma occurring naturally in the cottontail rabbit (genus *Sylvilagus*) and transmissible to the domestic rabbit (genus *Oryctolagus*). Later, Rous and Beard (13) found that the experimentally induced papillomas in domestic rabbits often progressed to carcinomas; and Syverton and Berry (16, 17) observed the same phenomenon in cottontail rabbits. Because of its possible significance for the pathogenesis of mammalian cancers in general, this virus-induced papilloma-to-carcinoma sequence aroused wide interest and has been the subject of numerous investigations.

An outstanding feature of the Shope papilloma is the production of excessive amounts of keratinized epithelium, which may develop into enormous epithelial horns, sharply demarcated from the underlying tissue.[1] The present communication concerns some of the chemical properties of this "keratin" and also of carcinomas deriving from the papillomas in cottontail rabbits.

Keratinization, or cornification, which reflects the ultimate natural alterative change in superficially situated structures of ectodermal origin, yields a wide variety of hard anuclear entities, such as horns, hoofs, nails, hair, the outer layers of scales, feathers, and horses' burrs. The same process occurs excessively when structures of ectodermal origin are subjected to unusual stress. Thus callosities result from excessive physiological stress and hyperplastic keratoses from physical or chemical agents, such as prolonged exposure to sunlight (8), and from carcinogenic agents (9). More striking, perhaps because of the abnormal and irregular features, are the bizarre manifestations of the process of keratinization which occur during the pathogenesis of epitheliomas. For example, keratinized "pearls" are regularly present in cystic papillomas and acanthomas.

It has long been customary for histologists to employ "keratinization" as a descriptive term in the process of development of all corneous excrescences and to designate the resulting substance as "keratin," irrespective of whether the product was a horn, hoof, callosity, or nail. Modern chemical investigations, however, have made possible a separation into two main categories: eukeratin and pseudokeratin. Block and Vickery (2) revised the chemical criteria for the characterization of keratins when they stated: "A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalies, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine and arginine that the molecular ratios of these amino acids are respectively approximately as 1:4:12." Subsequently, Block (4) was responsible for their separation when he presented evidence that "the proteins of ectodermal origin fall into two main groups, eukeratins and pseudokeratins. The eukeratins are insoluble and resistant to digestion by pepsin and trypsin and yield histidine, lysine and arginine in molecular ratios of approximate'y 1:4:12. The pseudokeratins appear to be somewhat more soluble and less resistant to enzymatic digestion and yield relatively more histidine and less arginine than the eukeratins."

The pseudokeratins analyzed at that time contained histidine, lysine, and arginine in average molecular ratios of 1:3:3. In more recent studies, ratios of 1:4:6 were found (6), and it was also stated that "from 25 to 60 per cent of the peudokeratins is often dissolved by treatment with pepsin and trypsin." According to the foregoing criteria, representative eukeratins are various animal hairs, fingernails, and cattle horn, and representative pseudokeratins are human skin, neurokeratin, and horses' burrs.

The present experiments indicate that the horny excrescence formed in rabbit papillomatosis

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† Now located at the School of Medicine, University of Minnesota, Minneapolis, Minnesota.

[1] As examples of the size attained by virus-induced papillomas on the ears of rabbits, we have observed two specimens that measured 13 cm. in length and more than 3 cm. in diameter and two others that measured more than 10 cm. in diameter and 6 cm. in length.

(Shope) must be classified with the pseudokeratins: its water-insoluble protein was from 32 to 51 per cent soluble in sodium hydroxide at pH 12; was insoluble in hydrochloric acid at pH 1; was from 57 to 87 per cent digested by pepsin at pH 1.8 or trypsin at pH 7.9; and contained histidine, lysine, and arginine in average molecular ratios of 1:2.3:2.5.

For comparison, specimens of the carcinomas that arose secondary to the initial virus papillomas in 6 cottontail rabbits were analyzed for the basic amino acids, to determine whether these occurred in ratios that would characterize the tissue. On the average, these materials contained relatively less histidine and a slightly higher proportion of lysine to arginine, the average molecular ratios of histidine:lysine:arginine being 1:3.3:3.1. These values were not deemed, however, to differ significantly from those found for the papilloma pseudokeratin.

EXPERIMENTAL

Papilloma.—The keratinized portions of virus papillomas (Shope) experimentally induced on domestic or cottontail rabbits were dissected as completely as possible from the mesodermal tissues. Each preparation comprised specimens from 6 to 9 rabbits. Preparation 2, from cottontail rabbits, was dried *in vacuo* over phosphorus pentoxide immediately after collection; the moisture content was 27 per cent. The other preparations were stored in acetone until used, when the acetone was drained off and the tissue dried *in vacuo*, ultimately over phosphorus pentoxide. The dried samples were comminuted in a meat-grinder and were separated by a 40-mesh screen into fine and coarse fractions.

Ether-extracted samples were prepared in a Soxhlet apparatus. For water extraction, samples were stirred for 15 minutes in 25 volumes of water, while heating in a boiling water bath, and were then collected by centrifugation. This treatment was repeated three times, after which the material was washed with acetone and dried *in vacuo*.

Carcinoma.—Each preparation comprised material from a single cottontail rabbit. Numbers 1 through 4 were primary carcinomas that developed at the site of experimentally induced virus papillomas. Number 5 consisted of metastases in the iliac lymph nodes; No. 6, of metastases in the lungs. The specimens had been stored for various periods of time in a refrigerator in 50 per cent glycerol and buffered at pH 7.0, under vaseline seal. Each was extracted three times with approximately fifty times its volume of ethyl alcohol, then treated similarly with ether, and finally dried *in vacuo*.

Solubility of crude papilloma keratin.—The "fine" fraction of preparation No. 2 was used without further treatment. It contained 1.4 per cent of ash, calculated as sodium, and 14.7 per cent of nitrogen (all determinations of nitrogen were made by a micro-Kjeldahl procedure), calculated on the anhydrous, ash-free basis. Then 250-mg. samples of the air-dry keratin (=33.9 mg. of nitrogen) were extracted for 24 hours at 23°–26° C. with 25.0-ml. portions of distilled water, 0.10 *N* sodium hydroxide, and 0.10 *N* hydrochloric acid, respectively. The extracts were clarified in an angle centrifuge and analyzed for total and nonprotein nitrogen, i.e., nitrogen soluble in 5 per cent trichloracetic acid. Protein nitrogen was calculated as the difference between total nitrogen and nonprotein nitrogen. The results, summarized in Table 1, showed

TABLE 1

SOLUBILITY OF CRUDE PAPILLOMA KERATIN

	NONPROTEIN NITROGEN DISSOLVED		PROTEIN NITROGEN DISSOLVED	
SOLVENT	Mg.	Per cent*	Mg.	Per cent†
Water	4.85	14	2.83	11
0.1 *N* NaOH	7.52	22	12.7	47
0.1 *N* HCl	6.75	20	6.28	23

* Percentage of the total nitrogen of the keratin.

† Percentage of the total protein nitrogen of the keratin. Total nitrogen of sample = 33.9 mg. Total nonprotein nitrogen of sample = 7.1 mg. Total protein nitrogen of sample (by difference) = 26.8 mg.

that 11 per cent of the protein of the crude tissue was soluble in distilled water, 47 per cent in the sodium hydroxide, and 23 per cent in the hydrochloric acid. Twenty-one per cent of the nitrogen of the tissue was nonprotein, a quite high value, which suggests tissue breakdown (14).

Digestibility of crude papilloma keratin.—A portion of 0.500 gm. of the fine fraction of preparation No. 2 was treated with trypsin (4 $[TU]^{cas\ S}$ [12] of Fairchild trypsin purified according to the method of Anson and Mirsky [1]) in 30.0 ml. of 0.1 *M* phosphate buffer at pH 7.8. Another portion of 0.500 gm. was treated with 0.050 gm. of pepsin (Merck, U.S.P.) in 30.0 ml. of 0.050 *N* hydrochloric acid. As controls, samples of casein (Kahlbaum, prepared according to Hammarsten) were treated in the identical manner. Digestion was carried out at 37.5° C., sterility being maintained by thymol. Samples were withdrawn immediately and after 1, 3, and 22 hours, and were analyzed for nonprotein nitrogen. The results, summarized in Table 2, showed that the keratin was extensively digested by the enzymes and, in the case of pepsin, at the same rate as casein. The more rapid digestion of casein by trypsin was probably attributable to the fact that the casein was in solution, whereas with

pepsin the undigested casein, like the keratin, remained insoluble.

Solubility and digestibility of ether- and water-extracted papilloma keratin.—Since preliminary experiments had shown that the crude papilloma keratin contained considerable lipid and water-soluble material, additional tests of solubility and digestibility were made on preparations of the tissue that had been subjected to extraction with ether and hot water (4). One hundred-milligram samples of the keratin, dried to constant weight at 100° C. *in vacuo* over phosphorus pentoxide, were extracted for 48 hours at room temperature, respectively, with 10.0 ml. of 0.1 *N* NaOH (final pH = 12.0) and 10.0 ml. of 0.15 *N* HCl (final pH = 1.0); and at 37.5° C. with 10.0 ml. of 0.5 per cent aqueous pepsin solution (Merck, U.S.P., initial pH = 1.9) and 10.0 ml. of 0.5 per cent aqueous trypsin solution (Fairchild, initial pH = 7.9). The enzymatic digestions were protected by thymol against contamination. The residues were collected in an angle centrifuge, washed three times in from 10 to 15 ml. of water, and dried to constant weight at 100° C. *in vacuo* over phosphorus pentoxide. (The weight of the sample *minus* the weight of the residue equals the weight of material dissolved or digested, respectively.)

The results, which are summarized in Table 3, confirmed the findings with the crude keratin. Although the water-insoluble material was not soluble in the hydrochloric acid, it was from 32 to 51 per cent dissolved by the sodium hydroxide and was from 57 to 87 per cent digested by the enzymes.

Determination of basic amino acids.—In an early experiment, papilloma preparation No. 2 was analyzed by Block's procedure (5) for histidine, lysine, and arginine, which were found in respective molecular ratios of 1:4.2:2.9 (Table 4), values approximating those that had been reported for pseudokeratins (6).

With one exception, to be discussed presently, all other determinations of the basic amino acids were carried out by the microbiological method of Stokes, Gunness, Dwyer, and Caswell (15). Each value recorded (Table 4) represents the mean of values determined in duplicate or triplicate at each of two or more levels of the sample and, in three-fourths of the cases, the results of replicate runs. The results showed a surprising uniformity in the keratins tested, which comprised one batch from domestic rabbits (6d) and the "coarse" and "fine" fractions of a batch from cottontail rabbits (7d and 7e). The average molecular ratios—histidine: lysine: arginine = 1:2.3:2.5—again approximated those reported for pseudokeratins (4). Evidently the content of the basic amino acids reflected no generic difference between the keratins, nor did screening result in any fractionation. Furthermore, extraction with hot water did not change the molecular ratios (preparations $6d_w$, $7d_w$, $7e_w$), although the residual proteins did contain slightly increased absolute amounts of the basic amino acids.

TABLE 2

TRYPTIC AND PEPTIC DIGESTION OF CRUDE PAPILLOMA KERATIN AND OF CASEIN

	Trypsin						Pepsin					
	Keratin			Casein			Keratin			Casein		
Time (hours)	Total NPN* (mg.)	NPN formed by digestion Mg.	% †	Total NPN (mg.)	NPN formed by digestion Mg.	%	Total NPN (mg.)	NPN formed by digestion Mg.	%	Total NPN (mg.)	NPN formed by digestion Mg.	%
0	26.6	....	..	22.5	....	..	21.1	....	..	10.4	....	..
1	51.0	24.4	36	83.4	60.9	86	51.8	30.7	45	44.0	33.6	47
3	60.8	34.2	51	84.6	62.1	87	59.8	38.7	57	50.6	40.2	57
22	70.4	43.8	65	84.3	61.8	87	68.9	47.8	71	62.0	51.6	73

* NPN = nitrogen soluble in 5 per cent trichloracetic acid.
† Percentage of the total nitrogen of the keratin or casein, respectively.

Comparative analyses of six papilloma-induced carcinomas revealed a fair degree of uniformity, histidine varying from 1.88 to 2.67 per cent, lysine from 5.75 to 7.26, and arginine from 6.17 to 8.10. Except for No. 1, the carcinomas contained relatively less histidine and a slightly higher proportion of lysine to arginine. The mean molecular ratios correspond relatively closely to those noted above for the keratinized portion of papillomas, a result that may reflect the common epidermal derivation of the two types of tissue.

Comparison of results with previously reported analyses of keratins.—In the first of the present series of determinations, a sample of ether-extracted hair from an albino rabbit was included as a control representing presumably a typical eukeratin. The amounts of arginine (8.80 per cent) and lysine (2.83 per cent) found were in good agreement with those reported in the most recent compilation of revised data on eukeratins (7), but the value for histidine (1.80 per cent) was nearly three times the mean value for six types of animal hair

(0.65 per cent). The basic amino acid content of rabbit hair has not been reported previously.

Because of the discrepancy in the histidine value, in later experiments measurement was made of the basic amino acid content of the same rabbit hair, of rabbit toenails (not previously reported), and of human fingernails (3), all prepared by repeated extraction with hot water and digestion by pepsin. This treatment effected a slight reduction in the histidine and increases in the lysine and arginine contents of the rabbit hair. Indeed, the arginine and lysine contents of all three specimens were somewhat higher than those previously reported for eukeratins, although the molecular ratio of arginine to histidine, 2.6, was in good agreement with the published figures, which ranged from 2.1 to 3.4, with a mean value of 2.8 for fourteen eukeratins (7). The histidine contents, however, were of the order of twice as great as expected.

Since the previously reported analyses of keratins for basic amino acids had nearly all been made by some variant of the classical Kossel-Kutscher procedure (11), it seemed important to analyze one of the present specimens by one of these methods. Accordingly, a pool of preparations $6d_w$, $7d_w$, $7e_w$ was analyzed by Block's procedure (5). The arginine value found was 90 per cent of that found by the microbiological assay; the lysine value, 96 per cent; the histidine value, only 75 per cent. Furthermore, the histidine fraction was not subjected to purification by mercury and copper treatments, which of themselves may result in an additional loss of 25 per cent of the histidine present (5).

Taken together, the foregoing results support the conclusion of Vickery and Winternitz (19) that the various modifications of the Kossel-Kutscher procedure give low values for histidine. That the results may be excessively low in proteins of low histidine content is indicated by published analyses of silk fibroin, whose histidine content was re-

TABLE 3

SOLUBILITY OF PAPILLOMA KERATIN

Material*	Preliminary treatment†	Solubility in 0.1 *N* NaOH	Solubility in 0.15 *N* HCl	Digestibility by Pepsin	Digestibility by Trypsin
		Per cent	Per cent	Per cent	Per cent
DR-papilloma $6d_w$	Ac, E, W	51	2	82	84
CR-papilloma $7d_w$	Ac, E, W	39	2	57	86
CR-papilloma $7e_w$	Ac, E, W	32	0	86	87

* DR, domestic rabbit; CR, cottontail rabbit.
† Ac, extracted with cold acetone; E, extracted with ether (Soxhlet); W, extracted with hot water.

TABLE 4

BASIC AMINO ACID CONTENT OF KERATINIZED PAPILLOMAS (SHOPE), OF CARCINOMAS AND OF SOME EUKERATINS

Material*	Preliminary treatment†	Histidine (%)	Lysine (%)	Arginine (%)	Molecular ratios Hist.	Lys.	Arg.	Arg./Lys.
CR-papilloma 2‡	None	1.48	5.93	4.77	1	4.2	2.9	0.7
DR-papilloma 6d	Ac, E	2.23	4.91	5.95	1	2.3	2.4	1.0
DR-papilloma $6d_w$	Ac, E, W	2.47	5.48	6.97	1	2.4	2.5	1.1
CR-papilloma 7d§	Ac, E	2.27	4.81	6.36	1	2.3	2.5	1.1
CR-papilloma $7d_w$	Ac, E, W	2.45	5.40	7.02	1	2.3	2.5	1.1
CR-papilloma 7e‖	Ac, E	2.34	4.64	6.16	1	2.1	2.3	1.1
CR-papilloma $7e_w$	Ac, E, W	2.49	5.42	6.90	1	2.3	2.5	1.1
CR-papilloma $6d_w$, $7d_w$, $7e_w$ pooled¶	Ac, E, W	1.85	5.20	6.28	1	3.0	3.0	1.0
CR-carcinoma 1	Al, E	2.67	5.75	8.00	1	2.3	2.7	1.2
CR-carcinoma 2	Al, E	2.10	7.26	8.10	1	3.7	3.4	0.9
CR-carcinoma 3	Al, E	1.88	6.94	7.61	1	3.9	3.6	0.9
CR-carcinoma 4	Al, E	1.90	5.96	7.16	1	3.3	3.4	1.0
CR-carcinoma 5	Al, E	1.93	6.81	6.17	1	3.7	2.8	0.8
CR-carcinoma 6	Al, E	2.27	6.55	6.94	1	3.1	2.7	0.9
DR-hair	E	1.80	2.83	8.80	1	1.7	4.4	2.6
DR-hair	E, W, P, W	1.67	3.15	9.60	1	2.0	5.1	2.6
DR-toenails	W, P, W	1.26	3.47	10.40	1	2.9	7.4	2.5
Human fingernails	W, P, W	1.06	3.33	10.40	1	3.3	8.7	2.6

* CR, cottontail rabbit; DR, domestic rabbit.
† Ac, Al, E, W, extracted with acetone, alcohol, ether, and hot water, respectively; P, digested with U.S.P. pepsin at pH 1.8 and 37° C. for 24–48 hours.
‡ Analyzed by Block's procedure (5). Histidine fraction purified by mercury and copper treatments. Histidine isolated as the flavianate. Duplicate determinations.
§ "Coarse" fraction.
‖ "Fine" fraction.
¶ 0.801 gm. of $6d_w$; 0.961 gm. of $7d_w$; 0.861 gm. of $7e_w$. Analysis by Block's method. Histidine fraction not purified by mercury and copper treatments. Histidine isolated as the nitranilate. Single determinations.

ported by Vickery and Block (18) to be 0.07 per cent, whereas Stokes *et al.* (15) and Guirard *et al.* (10), using different microbiological procedures, found 0.41 per cent.

SUMMARY

The horny excrescence formed in virus papillomatosis of the rabbit (Shope) appears to be a "pseudokeratin": its water-insoluble protein was from 32 to 51 per cent soluble in sodium hydroxide at pH 12; was insoluble in hydrochloric acid at pH 1; was from 57 to 87 per cent digested by pepsin at pH 1.8 or trypsin at pH 7.9; and contained histidine, lysine, and arginine in average molecular ratios of 1:2.3:2.5.

In comparison, the carcinomas that arose secondary to the initial virus papillomas contained relatively less histidine and a slightly higher proportion of lysine to arginine, the average molecular ratios of histidine:lysine:arginine being 1:3.3:3.1. The differences, however, are not deemed to be sufficient to characterize the carcinomatous tissue.

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Effects of an Antireticular Cytotoxic Serum on the Brown-Pearce Carcinoma of the Rabbit*

DAVID MOVITZ, M.D.,† OTTO SAPHIR, M.D., AND ALFRED A. STRAUSS, M.D.

(From the Department of Pathology,‡ Michael Reese Hospital, Chicago 16, Illinois)

The spleen, representing a dense portion of the reticulo-endothelial system, is apparently capable of exerting a defensive reaction to malignancy. The comparative rarity of primary splenic tumors and of metastases to the spleen (1, 2, 3, 4) directed attention to this. Metastases to the spleen occur only in association with diffusely distributed metastases involving several organs (5), although a recent interpretation of similar data emphasized no significance to this phenomenon (6). Emulsified splenic tissue has exhibited an inhibitory effect when mixed with tumor upon transplantation, particularly with splenic tissue obtained from animals either bearing or having recovered from a malignancy (7, 8, 9).

Of various homologous adult tissues grafted onto chick embryos simultaneously with heteroplastic tumor, only spleen or marrow prevented the usual growth of the tumor (10). The successful defense of the chick embryos to heteroplastic tumor implants appeared to be associated with the stromal reaction which consisted essentially of an infiltration of lymphocytes (10). Varying morphologic patterns of neoplasms, particularly their stromal relationships, reflect to some extent their biologic degree of malignancy (8, 11, 12). The importance of the stroma is further emphasized by some studies on the mechanisms of x-ray therapy of tumors (13, 14). Reticulo-endothelial reaction to malignancy is frequently observed in regional lymph nodes as follicle and/or reticulum-cell hyperplasia. With more extensive carcinomatous involvement, a more general reaction is often manifested by blood cellular changes, such as leukocytosis, with an increase in both the neutrophiles and monocytes and a decrease in lymphocytes, eosinophiles, and basophiles (15, 16).

Additional evidence of defensive reactions of the spleen and other parts of the reticulo-endothelial system to neoplastic growth is obtained from experiments with animals either splenectomized (17, 18, 19) or having their reticulo-endothelial system blocked by trypan blue or carmine (20, 21, 22, 23), as these procedures, properly done, were associated with either enhancement of malignancy or loss of tumor immunity.

Functionally, the reticulo-endothelial system is depressed in human beings and animals with malignancy, as is indicated by the Congo red index (24) or indirectly by the effect of administering certain extracts of their blood upon the Congo red index of normal test animals (25, 26, 27). This depression also occurs following successful tumor transimplantation (26), carcinogenic x-irradiation (28), or application of carcinogenic hydrocarbons (29). Noncarcinogenic hydrocarbons similarly applied do not depress the reticulo-endothelial dye-absorbing function (30).

To augment or stimulate the defensive reactions of the reticulo-endothelial system against malignancy, several general methods have thus far been used, including splenic tissue extracts (17, 31, 32, 33, 34, 35). Concentrated homologous normal spleen extracts (33), and particularly an extract of spleen from tumor-regressed animals (34), appear to be unusually effective. Heterologous splenic extract has been administered with success to human beings with basal cell carcinoma (35). Extracts of liver (35), fetal skin (36), or human connective tissue (37) have also been used.

A second general method augmenting the defensive reactions of the reticulo-endothelial system to malignancy has been the application of tumor antibodies. Homologous Brown-Pearce tumor antibodies, developed by intraperitoneal injections of a saline extract of the tumor, suppressed the growth of intramuscularly implanted tumor fragments incubated in the specific antibody-containing immune serum prior to their transplantation (38). Administration of a specific tumor antiserum to rats with Murphy rat lymphosarcoma resulted in a greater incidence of regressions (39).

Also, reticulo-endothelial mechanisms occurring in certain inflammations appear to be effective against a variety of lesions, including carcinoma. Various neoplasms in man have been reported to

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† Ira Frank Research Fellow in pathology.

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have regressed during or subsequent to acute bacterial infections, particularly erysipelas, as first observed by Fehleisen (40). Bacterial toxin treatment, consisting of various prepared mixtures of toxins of the *Streptococcus erysipelatus* and of the *Bacillus prodigiosus* (41, 42), has given way to the purified tumor-toxic fractions of the *B. prodigiosus* (43, 44) Experiments with the anti-tumor toxin of *Schizotrypanum cruzi* by Roskin have indicated that, by itself, it is not effective upon the tumor because trypan blue blockade of the reticulo-endothelial system rendered the toxin ineffective (45).

Attempts to stimulate the reticulo-endothelial system directly by a reticulo-endothelial cytotoxic serum followed certain fundamental developments. First was the elicitation and demonstration of tissue-specific serum antibody cytotoxins against tracheal epithelium of cattle by Von Dungern; these cytotoxins were found also to be species specific (46). Next Metchnikoff demonstrated that, with minute doses of a cytotoxin, stimulatory instead of cytotoxic effects may be obtained (47). More recently Chew, Lawrence, and Stephens observed marked depression of peripheral blood and tissue leukocytes following administration of a certain dosage of antileukocytic serum to guinea pigs (48). Finally, the application of established principles of serologic titration by complement fixation to a tissue antiserum was made by Marchuk (49), who did this with a combined spleen and marrow-tissue antiserum, designating it as "anti-reticular cytotoxic serum." Using this antiserum, Bogomolets reported that either an enhancement or an inhibition of carcinoma resulted, depending on whether relatively large or small doses were administered (50).

Pomerat and Anigstein, having produced a similar antiserum, confirmed by *in vitro* study both its tissue and its species specificity. Its specific inhibitory action on spleen tissue in culture occurred with dilutions of homologous antiserum up to 1:20 (51, 52). The Walker rat sarcoma 319 in conjoint tissue culture with splenic tissue grew unimpeded. This sarcoma in culture with the homologous antiserum grew unimpeded in serum dilutions above 1:4 but was impeded in serum dilutions up to and including 1:4. The addition of the antiserum to a conjoint tissue culture of spleen and the sarcoma, however, resulted in distinct tumor inhibition in as high serum dilution as 1:256 (53). Pomerat and Anigstein further observed that relatively large doses, 0.5 cc., of the reticulo-endothelial immune serum administered to *Bartonella* carrier rats resulted in a recurrence of the *Bartonella* infection, as also occurred with trypan blue blockade of the reticulo-endothelial system or by splenectomy (54).

Miale, studying dog spleen antiserum, observed upon its administration to dogs both a relative and an absolute increase of the mononuclear cells in the peripheral blood, which began 24 to 48 hours following the first injection and gradually returned to the base level within 20 days (55). Using the capacity for absorption of stain by histiocytes as an index of reticulo-endothelial activity, Provenzale observed that the greatest number of histiocytes per microscopic field were filled with stain in sections from spleen, marrow, liver, and lymph node from rabbits given antireticular cytotoxic serum, as compared with rabbits given an antimuscular cytotoxic serum or normal serum (56).

EXPERIMENTAL EFFECTS OF ARC[1] SERUM ON THE BROWN-PEARCE CARCINOMA

The antineoplastic properties of antireticular cytotoxic serum were investigated *in vivo* by us with rabbits implanted with Brown-Pearce carcinoma. This carcinoma has already been influenced by various procedures: immunity to the tumor was attained following intracutaneous tumor implantation (57); successful transplantation was prevented by first incubating the tumor fragments in immune serum (38); and its malignancy was lowered by exposing the tumor-bearing rabbits either to continuous illumination or to continuous darkness (58). Moreover, enhancement of its malignancy has been observed following administration of trypan blue (22), total ablation of the thyroid gland (59), or interruption of the cervical sympathetic chain (60). Abrogation of immunity to this tumor has been accomplished by trypan blue administration (23).

The Brown-Pearce carcinoma of the rabbit ordinarily exhibits a distinct natural variation in its degree of malignancy when transplanted into similar rabbits simultaneously, malignancy varying from rapidly fatal to slowly growing or even completely regressive (61). Thus this neoplasm lends itself to experimental changes. Furthermore, a well-mixed tumor suspension, with obviously identical growth potentialities throughout the suspension, when simultaneously transplanted into similar rabbits in equal quantity and into the same type of tissue (testis) will reflect the various degrees of resistance to the tumor by the resulting degree of malignancy in these rabbits. Though we used hybrid rabbits, the controls as well as the serum-treated were apparently similar hybrids.

[1] ARC = antireticular cytotoxic.

The validity of using hybrids is elucidated in the discussion.

The serum was made by the intravenous administration of increasing doses of normal spleen and marrow suspension-extract in saline to a dog at 5-day intervals until the complement fixation titer was at least 1:80 (62).

Nine consecutive experimental series were conducted, each consisting of ten or twelve hybrid male rabbits with positive intratesticular transplants of the Brown-Pearce carcinoma. The rabbits in each series were about equally divided into a serum-treated and a control group.

PROCEDURE OF TRANSPLANTATION

Tumor tissue was obtained under aseptic precautions through an abdominal incision from a previously transplanted rabbit. This was well minced and then emulsified through a tissue press. Sufficient sterile normal saline was added to render the emulsified tumor tissue adequately fluid for delivery through a syringe and an 18-gauge needle. The quantity of saline necessary varied somewhat. The ratio of tumor to saline in one series, for example, was 2.5 gm. of tumor to 1 cc. of saline.

The tumor suspension was injected into both testes of all the rabbits of a series, 2 cc. of the tumor suspension being injected into each testis. During the injection, a small quantity of the suspension was felt by palpation to be escaping into the peritoneal cavity. As a result, the transplantation was principally intratesticular and to a minor extent also intraperitoneal.

Following transplantation the rabbits were placed in their cages in the animal room, which had windows on two sides and was constantly ventilated. The diet consisted of liberal portions of hay, grains, and cabbage. The testes were examined daily until the tumor appeared grossly to be growing within at least one testis. This occurred in from 5 to 10 days. The rabbits with tumors were then separated more or less equally into a serum-treated and a control group.

The serum in the first six series was administered in three doses, at about 48-hour intervals, with each successive dose being approximately tripled in quantity. In the last three series each dose of serum was the same and was administered at 7- to 9-day intervals. Administration of the serum was begun as soon as the tumor appeared established, usually on the fifth to tenth day following the transimplantation, except for Series II and III. In Series II the serum was begun simultaneously with the transplantation, and in Series III the serum treatment was completed 1 week prior to the transplantation. All serum was administered subcutaneously in the back and, for accuracy of dosage, in dilutions of saline ranging from 1:10 to 1:1000, depending on the undiluted dosage which ranged from 0.0001 to 0.27 cc. The serum dilution was always made immediately prior to administration. The controls received no injections whatever.

The essential data consist of (1) the duration of survival following the tumor transimplantation (2) the level of malignancy as based on tumor distribution found at necropsy, and (3) percentage of regressions. Degrees or levels of malignancy of this tumor were originally noted by Brown and Pearce (61) and by Pearce and Van Allen (58, 60). They defined three general levels of malignancy, the more diffuse distribution of the tumor as the more highly malignant. In the course of our Series I, it became apparent that the distribution of the tumor among the controls differed markedly from that among the serum-treated. These serum-treated animals, which also had died earlier, presented a much more diffuse distribution of the tumor. Thereupon, need for detailed biologic grading of the malignancy became apparent and was set up accordingly. High degree of malignancy appears to consist of an orderly successive involvement of organs by the tumor, as indicated in Table 1, correlating fairly well with duration of survival.

All the rabbits dying with tumor presented involvement of at least the testes and the peritoneum. The peritoneal regions presenting tumor were, first, along one or both vasa, then upward upon the great omentum and, third, on the abdominal side of the diaphragm, the mesentery, the parietal peritoneum, and frequently the serosa.

TABLE 1

MALIGNANCY GRADING AS DETERMINED BY TUMOR DISTRIBUTION

Tissue involved	Level	Tissue involved	Level
Testes and peritoneum along vas	1	*Plus* lung (macroscopic)	6
Plus great omentum	2	*Plus* mediastinum	7
Plus parietal peritoneum (wall and diaphragm) and serosa	3	*Plus* myocardium and pericardium	8
Plus liver	4	*Plus* spleen	9
Plus kidney	5	*Plus* adrenal	10

These various involvements are graded 1, 2, and 3 (Table 1).

Microscopic involvement of the lungs was noted in practically all the rabbits, even with the relatively low malignancy levels of 3. Macroscopic lung nodules, however, were found only in those tumor rabbits with a much higher level of malignancy—in those with at least liver and kidney involvement also.

Involvement of the liver in addition to the peritoneal surface was graded 4. With the kidney involved additionally, a level of 5 was designated. If in addition to involvement of the preceding regions the lung presented gross tumor nodules, a level of 6 was designated. Higher levels are further indicated in Table 1. All organs were examined histologically.

Occasionally an organ in the successive order of malignancy levels was found not to be involved. The total level of malignancy was then considered to be one degree less. Very occasionally, two or three organs were "missed," and thereupon the grading was reduced by the number of levels omitted.

This grading is apparently consistent also with the observations of Foulds, who found that trypan blue administration to rabbits with Brown-Pearce carcinoma was followed by a much higher incidence of metastases to the lungs, liver, and spleen, particularly the latter (22). Also in man, when the spleen is involved with metastatic carcinoma, it is only in association with diffusely distributed metastases to other organs, such as lung and liver (5), indicative of a highly malignant state.

Differences between serum-treated and control animals are valid only within each series, inasmuch as the degree of malignancy of this tumor appears to vary with the season and the weather (61). Also, since the quantity of exposure to light appears to influence the degree of malignancy of the Brown-Pearce carcinoma (58), the rabbits in the two groups of each series (serum-treated and controls) were so arranged in their cages in the animal room that approximately equal intensity and duration of natural and artificial light fell upon all animals of each series. In each series one group of hybrid rabbits was set up against a similar control group. The narrow range of malignancy levels occurring in each series, particularly of the control groups, attests to the adequate control of the various experimental factors —even to the extent that often a distinct variation in the levels of malignancy did not occur.

Though the levels of malignancy, for convenience, are indicated in numerical terms (Table 1) and averages of groups are calculated with these numbers, differences in malignancy between rabbits or groups of rabbits are not at all quantitative but, rather, qualitative. Thus a level of 7 is not twice as malignant as 3.5 but indicates involvement of certain organs in addition to the peritoneal surfaces. Statistical analysis does not lend itself to qualitative differences, and thus small numerical differences are more significant than would be indicated on a quantitative basis. From another point of view the concept which is suggested is that of relative organ resistance or susceptibility to metastatic neoplasm.

From theoretical considerations gained from each experimental series and applied to succeeding series for their testing, it appears that the dosage of the serum plays an essential role in the result, thus apparently confirming the observations of Bogomolets (50), of Strauss, Horwitz, Levinthal, Cohen, and Runjavac (64), and of Miale (55), and lending further support to the original attempt by Metchnikoff to stimulate cellular proliferation with minute doses of a cytotoxin (47). The interval of administration of any particu'ar dosage also appears to be important.

SERIES I, II, AND III

In Series I, following establishment of the "testicular" tumors 9 days following transimplantation, four rabbits were given the antireticular cytotoxic serum and five served as controls. Three doses—0.03, 0.09, and 0.27 cc.—freshly diluted in 1:10 saline, were administered subcutaneously at 48-hour intervals. An apparent enhancement of the malignancy occurred following administration of the serum (Table 2). The administered dosages were thus considered cytotoxic.

In Series II the serum administration was begun 1 day after transplantation of the tumor. Half the twelve animals received three doses—0.01, 0.03, and 0.09 cc.—in 1:10 saline, at 48-hour intervals. This is one-third the dosage given in Series I, reduced because the previous dosage appeared cytotoxic, but still in the range of hundredths of a cubic centimeter. An apparent enhancement of the malignancy again occurred among the serum-treated (Table 3). The earlier administration of the serum, almost simultaneous with the transplantation, did not appear to alter its effects.

In Series III the three doses of serum were completed 1 week prior to the tumor transplantation. The dosages and intervals were the same as in Series II, and again a cytotoxic effect was apparently obtained, though less striking (Table 4).

SERIES IV, V, AND VI

Because the doses of serum in the first three series—0.01 to 0.27 cc.—were apparently cytotoxic and resulted in enhanced malignancy, the dosage of the serum in the next three series—Series IV, V, and VI—was reduced in general to one one-hundredth (1/100) of the previous quantities used.

In Series IV the transplanted tumors appeared established 10 days after the transplantation, and thereupon three doses of serum were given to half the animals at 48-hour intervals. The three doses were 0.00015, 0.0003, and 0.0009 cc., respectively, given in 1:1000 saline, freshly diluted. Again the serum-treated exhibited a higher malignancy.

Serum administration to half the rabbits of Series V was begun on the tenth day following the transplantation, at which time the tumors appeared established. Three doses—0.0003, 0.0006, and 0.0009 cc., respectively—were given subcutaneously at 3- to 4-day intervals in 1:1000 saline. No significant difference was noted between the serum-treated and the controls. The malignancy levels averaged 3.75 and 3.5 for the two groups. The average survival times in the two groups were 34.5 and 30.5 days (Table 5).

Similar management of ten tumor rabbits in Series VI again resulted in no significant difference between the serum-treated and the controls (Table 9).

In view of the experience with Series V and VI, the doses of the serum ranging from 0.0001 to 0.0009 cc. were considered too minute to have an effect and thus were considered to be noneffective doses. In contrast, the doses given in the first three series, ranging from 0.01 to 0.27 cc., consistently enhanced the tumor malignancy and thus were considered to be cytotoxic doses.

TABLE 2

SERIES I

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
104	7	20	112	3	26
107	7	22	102	3	27
111	7	24	108	3	43
101	7	27	106	3	44
			109	5	44
Average	7.0	23.2	Average	3.4	36.8

TABLE 3

SERIES II

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
121	8	16	118	3	17
117	6	21	126	3	17
122	6	21	120*	3	38
124	5	22	125	5	62
116	5	49	119	4	64
Average	6.0	25.8	Average	3.6	39.6

* Only rabbit not showing microscopic tumor foci in lung.

TABLE 4

SERIES III

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
129	6	23	139	5	68
132	5	38	137	Regressed	....
130	8	44	138	Regressed	....
131	Immune (natural)	....	136	Immune (natural)	....
Average	6.3	35	Average	5	68

SERIES VII, VIII, AND IX

A short time prior to the beginning of our Series VII, Strauss, Horwitz, Levinthal, Cohen, and Runjavac reported that a reticulo-endothelial cytostimulatory effect was obtained in the treatment of experimental fractures in rabbits with one administration of 0.00125 cc. of the antireticular cytotoxic serum (64). This dosage, 0.00125 cc., is approximately one-tenth more than each of the noneffective doses given in our Series IV, V, and VI and is approx mately one-tenth of each of the cytotoxic doses given in our Series I, II, and III. Because our problem apparently required counteraction to a more or less sustained process-carcinoma, more than a single administration of serum seemed necessary.

In Series VII two doses of the serum, 0.00125 cc. each, were given, the second 9 days following the first dose, both in 1:100 saline dilution. The first dose was administered on the seventh post-transplantation day, at which time the tumors appeared grossly established. Table 6, Series VII and Table 9 indicate the results, which show for the first time in this study a suggestion of a favorable effect by the difference in malignancy levels obtained with serum treatment.

In Series VIII three doses of serum were administered, each 0.00125 cc., at 7- and 9-day intervals, respectively. The first dose was given on the eighth day following the transplantation, at which time the "testicular" tumors became grossly established. The results again suggest a favorable effect of the serum. The malignancy level of 5.5 (serum-treated) as compared to 6.8 (controls) was associated with a favorable survival time of 40.2 days for the serum-treated as compared to 29.8 among the controls. Two regressions occurred in the serum-treated group and one among the controls (see Table 7, Series VIII, and Table 9).

In Series IX two doses of the serum, the first

TABLE 5

SERIES V

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
155	3	18	157	3	18
154	3	34	161	3	18
156	5	39	160	3	24
162	4	47	159	5	62
158	Regressed	....	163	Regressed	....
Average	3.75	34.5	Average	3.5	30.5

TABLE 6

SERIES VII

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
321	5	17	328	5	15
326	3	19	324	7	19
323	4	20	320	7	20
327	5	21	329	7	21
319	3	24	322	8	22
			318	5—	26
Average	4.0	20.0	Average	6.5	20.5

TABLE 7

SERIES VIII

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
341	6	32	336	6	17
342	6	32	331	7	20
339	9	41	343	6	22
340	1	56	333	7	38
337	Regressed	....	334	8	52
338	Regressed	....	335	Regressed	....
Average	5.5	40.2	Average	6.8	29.8

at 7 days post-transplantation, and the second dose 9 days later, were given to the serum-treated animals. Each dose was 0.001 cc., given in 1:100 saline dilution. A slightly less malignant course occurred among the serum-treated, as indicated by an average 5.2 malignancy level for the serum-treated as compared to 6.1 for the controls, by an average survival time of 27.7 days among the serum-treated and 21.5 days for the controls, and by two regressions occurring among the serum-treated, whereas no regressions occurred among the controls of this series (Table 8, Series IX; Table 9).

In summary, in the first three series, the dosages of serum administered ranged from 0.01 to 0.27 cc. and controls were 3.5 to 4.0, and the survival times of each group within each series were similar (Table 5 and Table 9). This dosage was considered to be ineffective.

Continuing by trial and error with respect to dosages and intervals in treatment, the dosage of the serum in Series VII, VIII, and IX was increased up to one-tenth of the cytotoxic doses, 0.001 cc., and in addition was administered only every 7 to 10 days instead of at 48-hour intervals. An apparent tumor-inhibitory effect was observed in the serum-treated groups in these last three series (Table 9). In Series VII the serum-treated malignancy level was 4.0 as compared to 6.5 for

TABLE 8

SERIES IX

Serum-treated			Controls		
Rabbit no.	Malignancy level	Days of survival	Rabbit no.	Malignancy level	Days of survival
352	4—	19	350	6—	16
353	5—	19	358	5	16
354	9	24	349	6	21
356	3	49	361	7	21
351	Regressed	....	363	6	21
355	Regressed	....	362	7	34
Average	5.2	27.7	Average	6.1	21.5

TABLE 9

SUMMARY

Serum dosage	Series	Malignancy level		Days of survival		Regressions (per cent)	
		Serum-treated (average)	Controls (average)	Serum-treated (average)	Controls (average)	Serum-treated (average)	Controls (average)
0.01 to 0.27 cc.	I	7.0	3.4	23.2	36.8	0	0
	II	6.0	3.6	25.8	39.6	0	0
	III	6.3	5.0	35	68	0	66
0.00015 to 0.0009 cc.	IV	5.0	3.3	24	40.3	40	40
	V	3.75	3.5	34.5	30.5	20	20
	VI	3.5	4.0	26	24.6	60	40
0.001 cc.	VII	4.0	6.5	20	20.5	0	0
	VIII	5.5	6.8	40.2	29.8	33	16
	IX	5.2	6.1	27.7	21.5	33	0

Each serum-treated rabbit was given three increasing doses, at 48-hour intervals. An enhancement of the malignancy occurred with these dosages, as is summarized in Table 9. In each of these first three series a higher level of malignancy and a shorter survival time were noted in the serum-treated groups. Hence these dosages were considered cytotoxic, or too large.

Therefore, in the next three series—Series IV, V, and VI—approximately one one-hundredth of the previous quantities of serum was administered, the doses ranging from 0.0001 to 0.0009 cc. Except for Series IV, in which the serum-treated again exhibited an enhanced malignancy, no significant difference between the serum-treated and the controls was noted (Table 9). In Series V and VI the levels of malignancy for both serum-treated the controls. In Series VIII the serum-treated level was 5.5 as compared to 6.8, and the survival time of the serum-treated also was comparatively favorable, 40.2 days as contrasted to 29.8 days for the controls. In Series IX the malignancy level of the serum-treated was 5.2 as compared to 6.1 for the controls. The difference in survival time, though small, is also in favor of the serum-treated, 27.7 to 21.5 days. Furthermore, the regression rates in Series VII and VIII also tend to suggest a less malignant course following administration of the serum.

DISCUSSION

Our results would seem further to substantiate two concepts. First, neoplasia may be affected by reticulo-endothelial tissue, inasmuch as adminis-

tration of a reticulo-endothelial tissue antiserum, previously shown to affect reticulo-endothelial tissue (51, 52, 55, 56) affected the malignancy of the Brown-Pearce carcinoma. Second, a cytotoxic serum may be given in doses that apparently produce cytostimulation instead of cytotoxic effects, although, essentially, a tissue antiserum is cytotoxic and its biologic identification in the past has depended on its cytotoxic exhibition. It is clearly realized, however, that direct observation of any reactions of the reticulo-endothelial system to the antiserum were not made. The reactions are presumed because cited evidence indicates an inverse relationship between stimulative reaction of the reticulo-endothelial cells and tumor growth, i.e., enhanced malignancy being associated with depression of this tissue system and, conversely, inhibit on of the tumor being associated with a stimulated reaction of the reticulo-endothelial system. In corroboration, the larger doses would therefore be depressing or cytotoxic (associated with enhanced malignancy), and the smaller doses would be related to the stimulation of this system and associated tumor inhibition. This appears to have occurred.

The cytotoxic effect (enhancement of malignancy) was more easily and clearly obtainable in our various series than was the cytostimulatory effect (tumor inhibition, as suggested by the results of our last three series). The difficulty in obtaining the presumed cytostimulatory effect of the reticulo-endothelial system on this carcinoma may be surmised from the fact that many and widely distributed nodules were present rather than a single lesion or one with just a few metastases. The relatively short duration of the animal's life with this tumor also indicates its high malignancy. Thus it would seem that a cytotoxic effect of this serum, with further depression of the reticulo-endothelial function upon its administration, would be markedly accentuated because of the extensive carcinomatous involvement and, conversely, that an attempt to stimulate the functions of this tissue system would be hampered by the multiplicity of the tumor lesions.

The levels of malignancy among the controls varied somewhat from time to time. In the course of the experiments during 1946 the levels among the controls were, in general, rather low, varying from 3.3 to 4.0, but mostly between 3.3 and 3.6. Beginning with the fourth series, higher levels of malignancy occurred among the controls and continued so for the remainder of the several series, varying from 6.1 to 6.8.

The use of a pure breed of rabbits would seemingly reduce the average range in the natural variations of malignancy of the Brown-Pearce tumor. A pure strain of rabbits, however, was not available. Casey observed variations in the degree of malignancy of the Brown-Pearce tumor, by statistical averages, between a number of different pure strains of rabbits (63). However, a considerable range of variation in the malignancy within each pure strain occurred, as indicated in Fig. 1 of Casey's report (63). Gorer observed that a mouse sarcoma which had arisen in a pure-breed line required very specific genes for its progressive growth, which, consequently, limited its successful transplantation to this particular pure breed or to certain of its hybrids (66). In contrast, Casey's experience indicates that the Brown-Pearce tumor, arising presumably in a hybrid ("albino male of stocky build"), was readily transplantable into, and lethal for, almost all varieties of pure strains (63) and hybrid rabbits. This would indicate a widespread occurrence of the particular genes necessary for the progressive growth of the Brown-Pearce tumor, although these have not as yet been determined. These widely occurring genes would also tend to obviate the necessity for using pure strains. The hybrids used in our various series appeared to serve as excellent controls, exhibiting insignificant variations, particularly as regards the narrow range of the degrees of malignancy as noted in the controls of the several series. Since Gorer has shown, by transplantation with the sarcoma arising in a pure-breed mouse, that some of the genes necessary for the progressive growth of a transplanted tumor may be identical with those determining blood groupings (66), it is possible that determinations of blood groups would be helpful in explaining some variations in the degree of malignancy among the different animals of a species.

Another *in vivo* demonstration of obtaining opposite effects with identical reticulo-endothelial tissue antiserum simply by varying the dosage was made in a study of this serum on experimental bone fractures in rabbits by Strauss, Horwitz, Levinthal, Cohen, and Runjavac (64). The quantity of serum which stimulated healing of fractures was 0.00125 cc., given once. This identical dosage, though repeated once or twice at 7- to 10-day intervals, was the same which we found apparently to stimulate reticulo-endothelial processes against malignancy.

Rogoff, Freyburg, Powell, and Rice reported that no effect of antireticular cytotoxic serum was obtained when it was administered to rats with induced pleuropneumonia arthritis (67). Inasmuch as the size of the dosage appears to be a critical factor, it is interesting to note that the dosage of

the serum that they employed, 0.00015 cc., was the same dosage with which we also could not, in general, detect a difference between the serum-treated and the controls (Ser. V and VI).

The variations in levels of malignancy within each group (serum-treated and controls) was small, fluctuating only slightly. However, marked variations in the levels of malignancy in Series VIII and IX occurred among the serum-treated. This might be interpreted as the result of the dosage of serum being in a critical zone between cytostimulatory and cytotoxic, in accordance with each individual animal's reticulo-endothelial reactivity to the amount of serum given. This would indicate that a reticulo-endothelial functional guide, similar in principle to the Congo red index test, might be helpful in determining the individual dosage necessary to obtain a reticulo-endothelial cytostimulatory effect.

SUMMARY

Administration of a reticulo-endothelial tissue antiserum, previously known to affect reticulo-endothelial tissue directly by *in vitro* tissue culture, was followed by definite effects on the degree of malignancy of the Brown-Pearce carcinoma *in vivo*. Reticulo-endothelial cytotoxic serum may be given in doses that apparently produce stimulation instead of a cytotoxic effect. The cytotoxic effect of the serum on the Brown-Pearce carcinoma was indirectly and presumably well demonstrated in the first three series by producing definitely enhanced malignancy. A dose too minute to have an effect was observed in Series V and VI. Finally, a mild cytostimulatory effect was obtained with one-tenth the cytotoxic dose, given at 7- to 10-day intervals, as indirectly indicated by the resulting tumor inhibition.

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In vitro Studies on the Effect of Spleen, Striated Muscle, and Kidney upon the Growth of Sarcoma 180 and Mammary Carcinoma of Mice*

LOGAN O. JONES, M.D.

(From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York)

The rarity of metastatic cancer to spleen, striated muscle, and kidney has been repeatedly noted in the past (1, 7, 29, 40, 63, 76), a fact which suggests that the malignant cell meets unknown influences antagonistic to its survival and proliferation in such sites. Cowdry has recently commented once again on this enigma, adding: ". . . it would be logical to try the effects of extracts of these tissues [spleen, striated muscle, and kidney] on experimental cancers . . ." (23).

Approaches to this problem employing *in vitro* technics have not demonstrated any tumor inhibition due to splenic influences. Stevenson (67), Danchakoff (24), Lumsden (50), and Wylegschanin (75) have reported observations on growth of neoplastic and splenic tissues in close proximity, either in tissue culture or following implantation into developing chick embryo membranes. In the several rodent carcinomas and sarcomas studied, tumor growth and cytology were not influenced by splenic explants from several mammalian sources, including the tumor donor strain of animals. The fact that Rous chicken sarcoma grows well in tissue culture with spleen (28) may not be germane to this problem, since a virus etiology has now been established for this tumor.

The several *in vitro* observations on concomitant growth of neoplastic and striated muscle tissue are not in agreement. Centanni (20) noted growth inhibition of a mouse carcinoma when skeletal muscle taken from tumor-resistant mice was added to cultures of this tumor but could detect no growth suppression upon addition of skeletal muscle explants from susceptible mice. In contrast, Wylegschanin (75) reported no antagonistic effects due to skeletal muscle in a similar study on a sarcoma and a carcinoma from rodent hosts. *In vitro* growth suppression of a rat sarcoma by several digests of bovine heart muscle was observed by Roffo (55). Since this worker could detect no similar effects from fresh muscle extracts from the same source, he attributed the observed inhibition of tumor growth to the presence of free amino acids in his muscle digests.

There are few recorded results on tumor growth in tissue cultures to which kidney explants have been added. Neither Lumsden (50) nor Wylegschanin (75) could detect alterations of tumor histology when a carcinoma and several sarcomas from rodent hosts were cultivated with renal tissue from tumor-resistant rodents.

These *in vitro* findings give no hint as to why cancer apparently metastasizes so infrequently to spleen, striated muscle, and kidney, and they do not lend support to the many favorable reports of animal and human malignancy treated with various organ extracts. The fact that such observations continue to appear in the literature may indicate that some workers are unaware of negative *in vitro* and *in vivo* studies in the past. It seemed of interest to reinvestigate these older tissue-culture observations and to supplement them by inoculating susceptible hosts with tumor explants after they had been cultured with these various tissues, in order to note *in vivo* tumor growth as well. To our knowledge, such latter observations have been made previously only by Stevenson (67) in the case of tumor exposed to splenic tissue *in vitro*.

MATERIALS AND METHODS

The studies were made on two commonly employed mouse tumors: sarcoma 180 from a Swiss mouse host, and a spontaneous adenocarcinoma of the breast obtained from mice of the Paris strain. Control biopsies were obtained in each case for future comparison. Spleen, skeletal muscle, and kidney from the same strain as the respective tumor donors were used for culture.

The roller-tube tissue-culture technic of Gey was selected to allow intimate contact of tissue juices from organ explants with growing tumor explants. A few hanging-drop preparations, using

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the "flying cover slip" method of Maximov, were also made; and, when growth in such preparations was found to differ in no detectable manner from that in the roller tubes, these hanging-drop cultures were used for making photographic records and permanent histologic whole mounts. In all studies, chicken plasma clots provided the matrix for growth; and chicken embryo extract, Tyrode's solution, and human placental serum mixtures supplied nutrient factors. Simms's ultrafiltrate of ox serum (60) was incorporated into the feeding solution of the hanging-drop preparations, to supply growth stimulants and to suppress fat globules.

Nonulcerated, firm tumors, approximately 2.5 cm. in diameter, from 10- to 14-week-old mouse hosts, were selected for tissue culture. For each tumor a total of 100 viable fragments, 2 mm. square, were planted, a quarter of which were grown in roller tubes in close proximity to spleen explants, another quarter with kidney explants, and a third quarter with skeletal muscle explants. Twenty-five explants of tumor and an equal number of each organ were grown separately to serve as controls. Because of the longer "lag-phase" of kidney and skeletal muscle, these explants were grown in culture for 4 to 8 days to insure good growth before starting the tumor cultures. Thus, for the neoplasms studied, the behavior of 200 explants of tumor was noted. Observations were made daily and photographic records of living and stained whole mount preparations were made at the end of 9 to 12 days. At the end of 10 to 12 days of tissue culture, sarcoma 180 tumor explants were injected into the dorsal subcutaneous tissues of the same strain as the tumor donor, and subsequent *in vivo* growth followed. Biopsy and histologic study were carried out after these tumors had reached approximately 2.5 cm. in diameter. These latter studies could not be carried out with explants of the spontaneous breast adenocarcinoma because susceptible hosts could not be obtained.

RESULTS

No observable alterations of tumor cytology or growth characteristics over the control observations were detected in the two malignant growths studied when grown in tissue culture adjacent to spleen, skeletal muscle, or kidney explants. Growth of the normal tissue explants which were adjacent to viable tumor cells resembled that of fragments of these tissues grown alone. No less than 84 per cent of tissue and tumor explants showed good growth in all series throughout the period of tissue-culture observations (see Table 1 and Figs. 1–6).

Following reinoculation of sarcoma 180 into susceptible hosts, the percentage of "takes" with tumor explants exposed to splenic and to renal tissue in culture was lower than the control figure observed when tumor fragments cultured alone were inoculated. These percentages do not differ significantly, however, by statistical analysis. It is possible that the figure of 30 per cent "takes" with the "splenic" tumor explants and the 46 per cent "takes" following animal inoculation with "renal" tumor would attain significance in a larger group of animals (see Table 1). Since the time of appearance, the rapidity of growth, and the histology of the tumors did not differ in the experimental and the control groups of animals, repetition of the studies, using larger numbers of mice, was not deemed worth while.

TABLE 1

THE EFFECTS OF SPLEEN, SKELETAL MUSCLE, AND KIDNEY ON TUMOR GROWTH

	Sarcoma 180				Mammary andenocarcinoma			
Tissue culture	Tumor alone	Tumor Spleen	Tumor Muscle	Tumor Kidney	Tumor alone	Tumor Spleen	Tumor Muscle	Tumor Kidney
Total explants	25	25+ 25	25+25	25+25	25	25+ 25	25+25	25+ 25
Per cent viable explants, 10–12 days	86	96+100	100+96	92+84	96	100+100	96+96	100+100
Growth	Good	Good	Good	Good	Good	Good	Good	Good
Cytology		Normal*	Normal*	Normal*		Normal*	Normal*	Normal*

Animal inoculations	Tumor alone	"Splenic" tumor†	"Muscle" tumor†	"Renal" tumor†
Number of animals	20	20	10	11
Per cent "takes"	65	30	90	46

* "Normal" connotes no observed difference from control organ or tumor in culture.
† Prefix "splenic," etc., refers to tumor explants grown with spleen, etc., in tissue culture.

DISCUSSION

Our inability to detect suppression of tumor growth *in vitro* attributable to the influence of spleen, skeletal muscle, or kidney is in essential agreement with the findings of previous workers. The influence of tissues of other mouse strains and of heterologous tissues was not observed, since the presumed antagonism exerted by spleen, skeletal muscle, and kidney exists in tumor hosts themselves, or metastatic cancer of these organs would

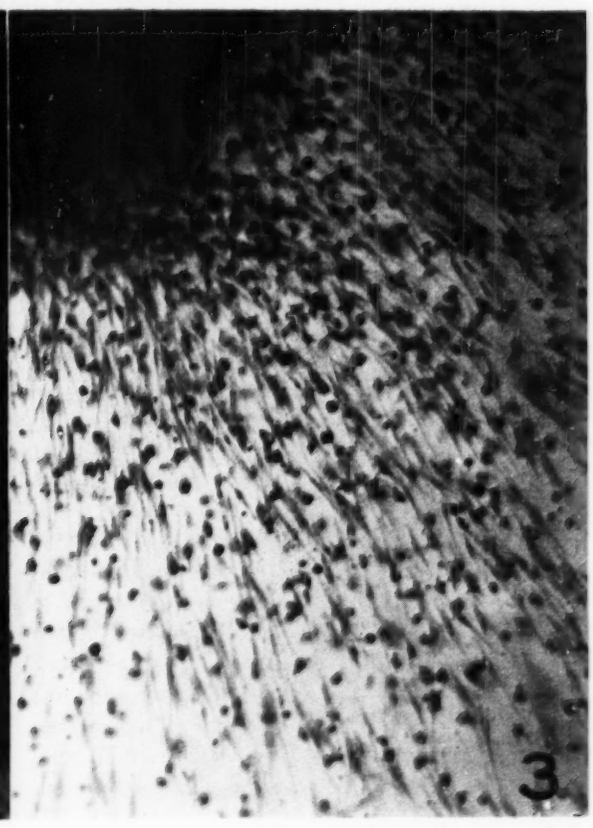
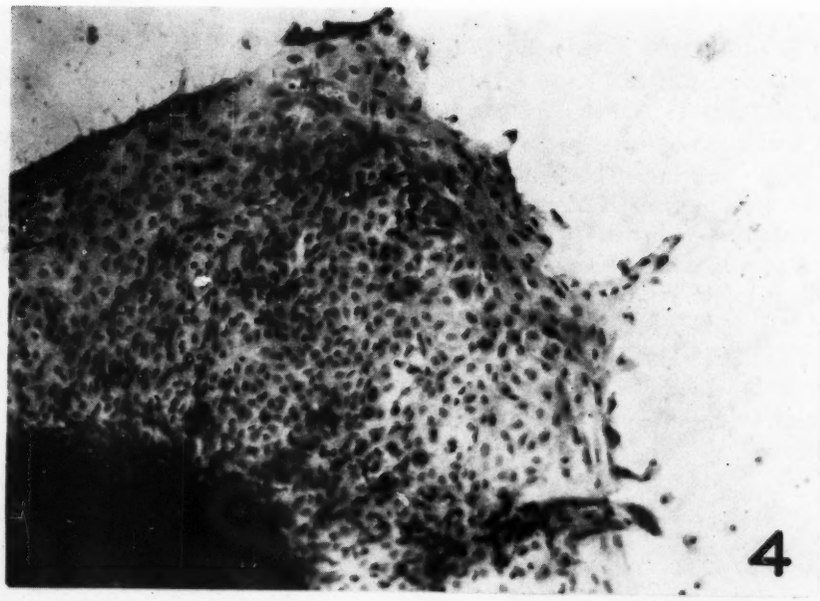
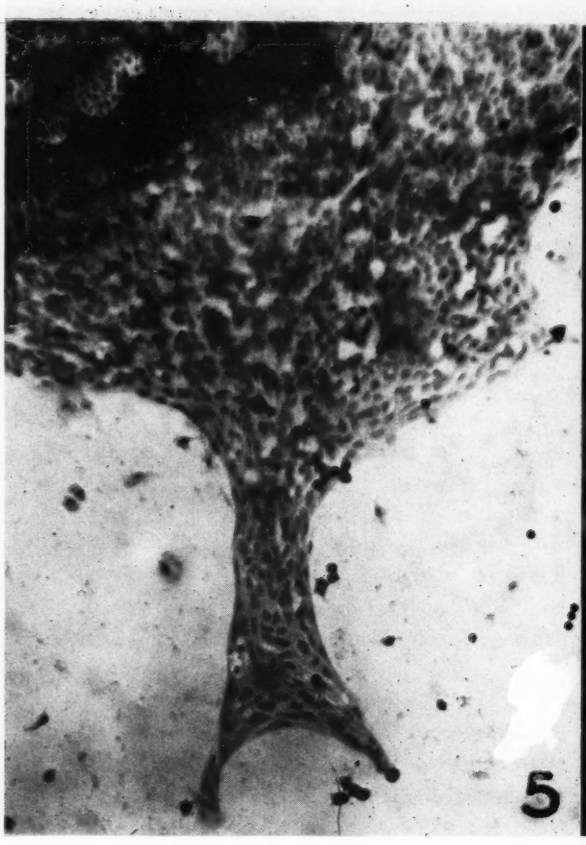


FIG. 1.—Sarcoma 180 grown alone for 10 days in tissue culture, during which time the explant was transplanted twice. Fixation in Bouin's and staining with Harris' hematoxylin. ×103.

FIG. 2.—Sarcoma 180 grown with spleen for 10 days in tissue culture, during which time it was transplanted twice. Fixation in Bouin's and staining with Harris' hematoxylin. ×103.

FIG. 3.—Mouse spleen grown with tumor depicted in Fig. 2. Fixation in Bouin's and staining with Harris' hematoxylin. ×103.





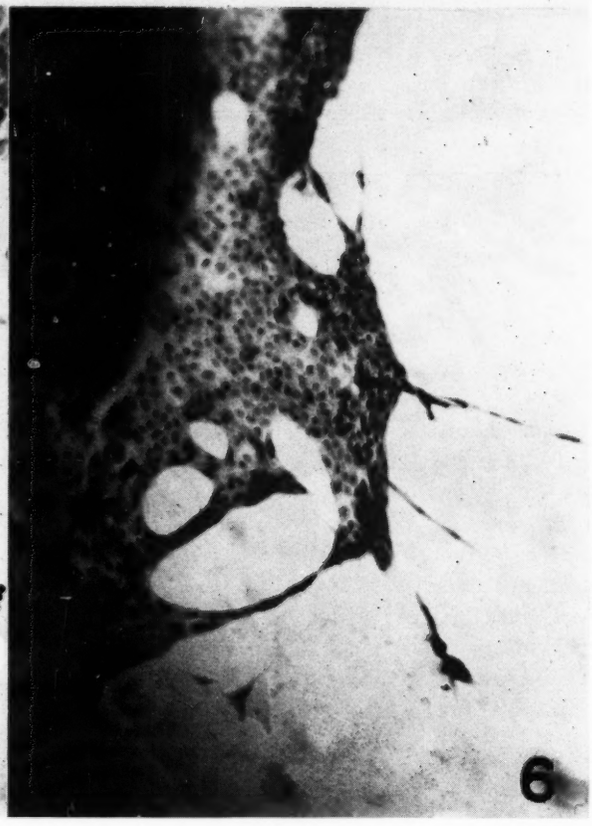


FIG. 4.—Spontaneous mammary adenocarcinoma of the mouse grown in tissue culture alone for 9 days. Fixation in Bouin's and staining with Harris' hematoxylin. ×103.

FIG. 5.—Spontaneous mammary adenocarcinoma of the mouse grown for 9 days in tissue culture with kidney. Fixation in Bouin's and staining with Harris' hematoxylin. ×103.

FIG. 6.—Mouse kidney grown with tumor depicted in Fig. 5. In this case the kidney was grown alone for 4 days to insure good growth, then transplanted at the time the tumor was planted. Fixation in Bouin's and staining with Harris' hematoxylin. ×103.

be more common. The enthusiastic reports of *in vivo* studies in which tissue extracts have been used as therapeutic agents may bear closer scrutiny in the light of *in vitro* studies, since we feel such enthusiasm has often been unwarranted. The use of tissue extracts in the chemotherapy of malignant disease will be presented only in outline form, in an attempt to interpret the rationale for their use and to analyze results. More extensive reviews (66, 71, 73, 74) of this subject bring out one fact worth emphasis at the start, namely, that consistently reproducible results have not been obtained with tissue derivatives. It is almost superfluous to add that no one of these preparations enjoys widespread clinical use—which is cogent testimony that they are inactive against malignant disease in humans.

A. Derivatives of Spleen and Reticulo-endothelial System

The use of splenic extracts in cancer therapy is inextricably bound to the use of various preparations to stimulate the reticulo-endothelial system, and this, in turn, to attempts to immunize actively and passively against cancer. Early reports by Woglom (70), Braunstein (13), and Levin (41) that splenic tissue implants and autolysates suppressed tumor growth in animals were essentially confirmed by others (32, 33, 34, 45). Subsequent attempts to prepare more potent compounds from splenic sources have continued to the present (2, 5, 10, 25, 46, 64, 69). It is worth emphasis that during such studies (25, 65, 69) stimulation of tumor growth and carcinogenic activity have been noted when splenic extract preparations were used.

The splenic hypertrophy and lymph node enlargement in some animals with malignant disease have been interpreted as activation of host defenses. Those proponents of the theory that the reticulo-endothelial system exerts suppressive influences upon tumor cells emphasize the frequency of splenic and lymph node hypertrophy in tumor hosts and point out that blockade of the reticulo-endothelial cells by foreign substances (3, 47, 56), their suppression by x-ray (51), or splenectomy (15, 52) has been associated with decreased animal resistance to cancer. Caspari (19), Braunstein (14), Fichera (27), and Bogomolets (11) have claimed that stimulation of the reticulo-endothelial system by their several preparations was an effective form of cancer therapy. These various observations require analysis. First, splenomegaly and lymphadenopathy are only inconstantly seen in malignant disease. When present, plausible coexistent infectious causes for reticulo-endothelial hypertrophy in tumor-bearing hosts can frequently be demonstrated by bacteriologic methods (72). Second, we are unaware of any reports indicating that humans splenectomized for blood dyscrasias, infections of the spleen, or traumatic rupture of that organ have a higher incidence of cancer than does the general population. Third, several workers (31, 42, 53, 54) have carried out successful inoculation of the intact animal spleen with a variety of animal tumors. All the *in vitro* evidence on adjacent growth of splenic and tumor tissue previously outlined is contrary to the hypothesis that extracts of the spleen may suppress the growth of existent cancer in animals. Finally, the favorable clinical and laboratory reports of several workers using reticulo-endothelial stimulant preparations in malignancy have not been confirmed by others (6, 18, 61). From the data presented in a very recent report (62) the favorable conclusions seem unwarranted.

Because the reticulo-endothelial system has been considered to have a significant function in immune responses in infectious diseases, the phenomenon of acquired or natural resistance to cancer was studied in relation to this system. The inhibition of tumor growth by sera of immune animals reported by some (21, 35, 44, 48) could not be confirmed *in vivo* (57) or *in vitro* (39) by other workers. The enthusiastic reports (4, 9, 38, 43, 49, 64) of immunizing animals against malignancy by the use of various products of actual tumor origin find no confirmation by other workers (22, 30, 36). In the case of certain animal tumors for which a virus etiology seems assured, humoral antibodies have been detected capable of conferring cancer resistance (17, 26, 37, 58). That viral agents play a role in the genesis of human cancer or in the majority of animal tumors has not yet been demonstrated, however. Immunity or resistance to neoplastic disease cannot be attributed to the presence of circulating antibodies on the basis of the evidence now available. That antagonistic influences differing from true antibodies and originating in the reticulo-endothelial system are responsible for natural or acquired resistance to cancer has not been shown. No morphologic evidence of splenic tissue stimulation by proliferating tumors has been detected *in vitro*. These objections can be justly raised, we feel, to the use of various "tumor vaccines" and to antireticular cytotoxic serum (ACS).

B. Products of Striated Muscle

The evidence that striated muscle extracts act as cancerolytic agents is rather limited. Early work by Itami (34) suggested that cardiac muscle extracts but not skeletal muscle preparations were capable of immunizing animals against subsequent

tumor inoculation. Over a period of years Roffo and his associates employed various muscle derivatives in experimental cancer therapy. They observed (55) first that fresh muscle extracts were inactive but that an acid hydrolsate of bovine heart caused regression of sarcomatous and carcinomatous lesions in rodent hosts when injected into the tumor or at remote sites. Boyland later reported (12) that an orally administered acid extract of ox heart suppressed tumor growth in rodents. Roffo attributed the activity of his preparation to the presence of free amino acids, while Boyland presented evidence that certain organi bases were the active ingredients in his muscle digests causing tumor regression. Since Itami inhibited cancer growth by using watery extracts of cardiac muscle, his conclusions are weakened by reports of these later workers. His negative results with skeletal muscle extracts are in agreement with our *in vitro* observations. No explanation based on differences in chemical composition between cardiac and skeletal muscle can be offered to account for his observation of tumor inhibition by cardiac but not by skeletal muscle extracts. Available analyses of amino acid content of these two types of striated muscle show almost identical qualitative and quantitative results (8). Studies by Shear (59) and by Turner (68) in which alleged cancerolytic effects of various chemical compounds were reinvestigated do not support the hypotheses of those who had contended that free amino acids and certain organic bases in striated muscle were active cancerolytic agents. After *in vitro* studies of tumor growth in several types of media, Brues and his associates (16) attributed a minimal tumor-inhibiting effect to ethanolamine, an observation previously recorded by Boyland in his *in vivo* studies. For these reasons we submit that more work is needed before definite conclusions can be drawn as to the efficacy of striated muscle derivatives in the treatment of malignant disease.

C. Products of Kidney

When he mixed rodent carcinoma cells with renal extracts prior to animal inoculation, Frankl (32) noted poor subsequent growth of tumor. There seem to have been few similar studies until Sperti reported (64) that watery extracts of kidney tissue enhanced cancer susceptibility in mice. He further stated, however, that deproteinized, cell-free extracts from the same source were capable of immunizing animals against tumor proliferation. To our knowledge this work has not been repeated by others. The *in vitro* studies previously mentioned, including our own, have failed uniformly to detect the presence of antagonistic influences to tumor growth inherent in kidney tissue. It would seem that *in vivo* studies bear repetition or that the negative results of those who have unsuccessfully attempted them should be recorded.

Several facts seem worth emphasis in conclusion. First, there is a dearth of reports confirming the efficacy of various reticulo-endothelial stimulants and derivatives of spleen, skeletal muscle, and kidney in the therapy of cancer. When such products have been used in human malignancy, most attempts to corroborate initial enthusiastic reports have been unsuccessful. Second, in most of the laboratory work in the field of organotherapy of malignant disease, observations have been made on transplantable animal tumors. It is a commonly accepted fact among cancer research workers that such transplantable neoplasms often undergo spontaneous involution and regression, thus making interpretation of *in vivo* results more difficult. Lastly, all *in vitro* observations of which we are aware show no depression of tumor growth by spleen or by kidney tissue. In the case of striated muscle extracts, *in vitro* observations are not in entire agreement. The allegedly low incidence of splenic, striated muscle, and renal metastases in malignancy remains unexplained.

SUMMARY

1. Mouse sarcoma 180 and spontaneous adenocarcinoma of the mouse mammary gland were grown in tissue culture with homologous spleen, skeletal muscle, and kidney from nontumor-bearing animals.

2. No tumor-growth effect was detected when such series were compared with tumor explants grown alone in tissue culture.

3. After tissue culture with spleen, skeletal muscle, and kidney, explants of sarcoma 180 produced malignant growths in mouse hosts histologically similar to control biopsies. The time of appearance and growth characteristics of these tumors did not differ from neoplasms appearing in control animals inoculated with tumor explants grown in tissue culture alone.

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Further Studies on the Pathogenesis of Ovarian Tumors in Mice*

MIN HSIN LI, PH.D.,† AND W. U. GARDNER, PH.D.

(From the Department of Anatomy, Yale University School of Medicine, New Haven, Connecticut)

The formation of granulosa-cell tumors and luteomas in intrasplenic ovarian grafts in castrated rats (1) and in intrasplenic and intrapancreatic ovarian grafts in castrated male and female mice has been reported previously (6–9). These experiments were based on two principles: (*a*) the capability of the liver to inactivate ovarian hormones when the hormones circulate through the hepatic portal system and (*b*) the increase of pituitary gonadotrophins subsequent to castration. It was assumed that the prolonged stimulation by augmented amounts of gonadotrophic hormones, to which the ovaries were subjected in these sites, was responsible for the neoplastic growths. Ovarian tumors were not observed in intrasplenic or intrapancreatic ovarian grafts with vascularized adhesions that permitted drainage through other than the hepatic portal system, or in female hosts that showed regular estrous cycles. Ovarian grafts in subcutaneous tissues or testes of intact and castrated mice did not readily become tumorous.

The present experiments are concerned with the influence of gonadal and gonadotrophic hormones in the development of tumors in intrasplenic ovarian grafts in castrated mice. The purpose of the investigation was to obtain additional evidence on the possible role of gonadotrophic hormones in the genesis of ovarian tumors.

TABLE 1

EFFECTS OF GONADAL AND GONADOTROPHIC HORMONES ON TUMORIGENESIS IN INTRASPLENIC OVARIAN GRAFTS IN MICE

GROUP	HOSTS	NO. OF MICE	TREATMENT	TYPE OF OVARIAN TUMOR AND AGE RANGE OF THE GRAFTS: Granulosa-cell	Luteoma	Mixed	Number and age of nontumorous grafts
A	♂	13	None	10 (207–278)	0	2 (239, 261)	1 (187)
	♀	9	None	5 (246–296)	4 (212)	0	0
B	♂*	4	None	0	0	0	4 (138–276)
	♀*	6	None	0	0	0	6 (185–282)
C	♂	8	Estradiol 16 μgm/week	0	0	0	8 (225–255)
	♀	5	Estradiol 16 μgm/week	0	0	0	5 (160–265)
D	♂	8	Testosterone 1.25 mg/week	0	0	0	8 (192–243)
	♀	6	Testosterone 1.25 mg/week	0	0	0	6 (220–267)
E	♂	8	Progesterone 1 mg/week	5 (213–254)	0	2 (242, 253)	1 (193)
	♀	5	Progesterone 1 mg/week	3 (243–257)	1 (284)	1 (273)	0
F	♂	5	Gonadotrophin (PMS) 25 I.U./day	1 (236)	0	2 (205, 232)	2 (222, 268)
	♀	5	Gonadotrophin (PMS) 25 I.U./day	0	0	3 (205–278)	2 (264, 284)

* Unilateral castrates.

MATERIALS AND METHODS

Male and female mice of the A, C_3H, and C_{57} strains, and several groups of hybrid mice (A × C_3H and CBA × C_{57}) were used.[1] They were kept in an air-conditioned room and were fed with Purina Fox Chow and water. Gonadectomy and autoplastic or homoplastic grafting of an ovary into the spleen were performed in one-stage operations. Most animals were 1 to 3 months old at the time of grafting. Six groups of experiments were undertaken (Table 1). The steroid hormones were administered subcutaneously in oily solutions and the gonadotrophic hormones were injected daily in aqueous solution. All injections were started in mice bearing grafts that were 92 to 138 days old.

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† Anna Fuller Fund Fellow in anatomy. Now at the National University of Peking, Peiping, China.

[1] Dr. L. C. Strong supplied some of these mice.

One subcutaneous injection of the following hormones was made each week: 16.6 μgm. of alpha-estradiol benzoate in oil, 1.25 mg. of testosterone propionate in oil, or 1 mg. of progesterone in oil.[2] Daily subcutaneous injections of 25 I.U. of pregnant mare's serum (PMS) were made in other mice. The number of animals in the several groups of experiments are given in Table 1. The experiments extended for a period of 10 months.

Vaginal smears were obtained from ovariectomized mice at intervals during the experiments. At autopsy the ovarian grafts and visceral organs were examined and preserved for histological study. Some of the induced ovarian tumors were transplanted into other mice to determine their capacity for continuous growth in other hosts.

Gonadectomized Mice with Intrasplenic Ovarian Grafts

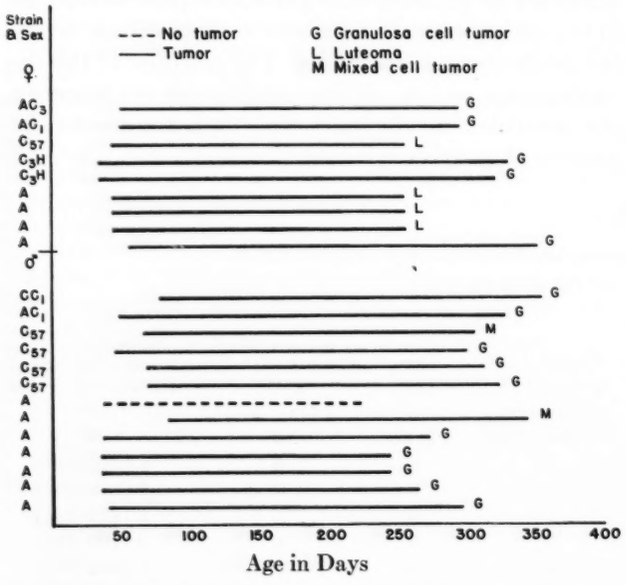

FIG. 1.—*A*, *C3H*, and *C57* refer to inbred strains of mice. The other symbols designate hybrid mice derived as follows: *AC1* (*A* ♀ × *C3H* ♂); *AC2* (*C3H* ♀ × *A* ♂); *AC3* (*C3H* low tumor ♀ × *A* ♂); and *CC1* (*C57* ♀ × *CBA* ♂).

OBSERVATIONS

A. Tumors in Intrasplenic Ovarian Grafts in Castrated Mice

Castrated males.—Ten granulosa-cell tumors and two mixed tumors—both granulosa and luteoma cells—developed in intrasplenic ovarian grafts in 13 castrated males (Fig. 1). These tumorous grafts ranged from 207 to 261 days of age, and the largest tumor measured 15 × 16 × 17.5 mm. (Fig. 4). The mouse that had no tumor of the grafted ovary died when the graft was 187 days old (Table 1).

The seminal vesicles and prostates of the castrated male mice bearing intrasplenic ovarian grafts were atrophic. The adrenal glands showed degenerate x-zones. No detectable hormonal effect on the kidneys and submaxillary glands was noted (8). In some animals the liver showed degenerate changes, as indicated by the large size of hepatic cells and the number of binucleated cells.

Castrated females.—Five granulosa-cell tumors and four luteomas developed in intrasplenic ovarian grafts in 9 castrated females (Fig. 1). These tumorous grafts ranged from 212 to 296 days of age, and the largest tumor measured 8 × 11 × 14 mm.

Irregular estrous vaginal smears were obtained during the latter part of the experiment in two ovariectomized mice bearing ovarian grafts that developed into granulosa-cell tumors. The liver of one (C_3H strain) of these two mice showed nodular regeneration, and its uterus weighed 135 mg. Cornified vaginal smears were not noted in the other 7 female hosts, and their uteri weighed, on the average, 45 mg. The adrenal glands showed degeneration of the x-zone.

Unilaterally Gonadectomized Mice with Intrasplenic Ovarian Grafts

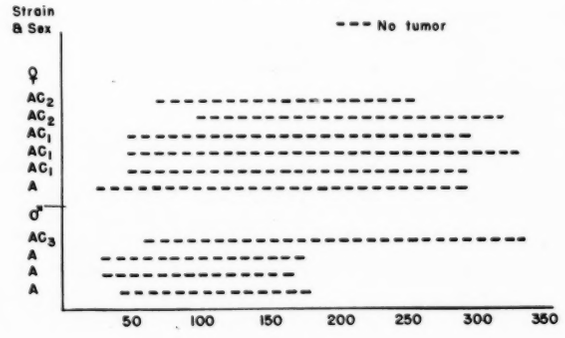

FIG. 2.—For meaning of symbols refer to Fig. 1

B. Ovarian Grafts in Unilaterally Castrated Mice

Ten unilaterally gonadectomized male and female mice carried intrasplenic ovarian grafts for 138 to 282 days (Fig. 2). The ovarian grafts remained small, about 2 to 3 mm. in size. Histologically, the grafts that resided in the female hosts were composed of small and large follicles and corpora lutea (Fig. 3). No hemorrhagic follicles or tubular ingrowths of the germinal epithelium were

[2] Grateful acknowledgment is made to Dr. E. Schwenk, of the Schering Corporation, for the alpha-estradiol benzoate (Progynon-B), testosterone propionate (Oreton), progesterone (Proluton), and PMS (Anteron); and to Dr. E. Oppenheimer, of the Ciba Pharmaceutical Products, Inc., for the progesterone.

noted. On the other hand, the ovarian follicles and interstitial cells formed the main structures of the ovarian grafts taken from the male hosts. Little lutein tissue was encountered. Ingrowths of the germinal epithelium were observed in small areas in the oldest ovarian graft in a male mouse.

The average uterine weight of the unilaterally castrated female mice was 105 mg. The intact testes and accessory genital organs in the males were maintained in a normal condition.

C. Ovarian Grafts in Estrogen-treated Castrated Mice

Castrated males.—No tumors were noted in intrasplenic ovarian grafts in 8 castrated males treated with an estrogen. The treatment was started 103 to 138 days subsequent to the grafting, and most of these hosts had received more than 10 weekly injections (Fig. 5).

The ovarian grafts were 3 to 4 mm. in diameter. They contained few to many small and medium-sized follicles. The stroma was composed of interstitial cells and, frequently, luteinized interstitial cells. Proliferation and tubular ingrowths of the germinal epithelium were observed in some areas in seven grafts, and some of the tubular structures were lined by simple cuboidal or columnar cells, as described previously (8). A few atretic and hemorrhagic follicles and corpora lutea were present in three grafts.

The urinary bladders of the male hosts were distended with urine. Effects of the estrogenic hormone were also indicated by the histological features of the seminal vesicles, prostates, kidneys, and submaxillary glands. The x-zones of the adrenal glands were absent, and groups of deeply stained small cells were noted in the subcapsular regions of the cortex of almost all adrenal glands examined.

Castrated females.—The intrasplenic ovarian grafts in 5 castrated females treated with an estrogen were nontumorous. The treatment was started 103 to 133 days subsequent to the grafting; 5 to 10 weekly injections were administered (Fig. 5).

Ovarian follicles and corpora lutea made up the greater part of three grafts that showed ingrowths of the germinal epithelial cells. A few hemorrhagic follicles were present in one of these grafts. Regres-

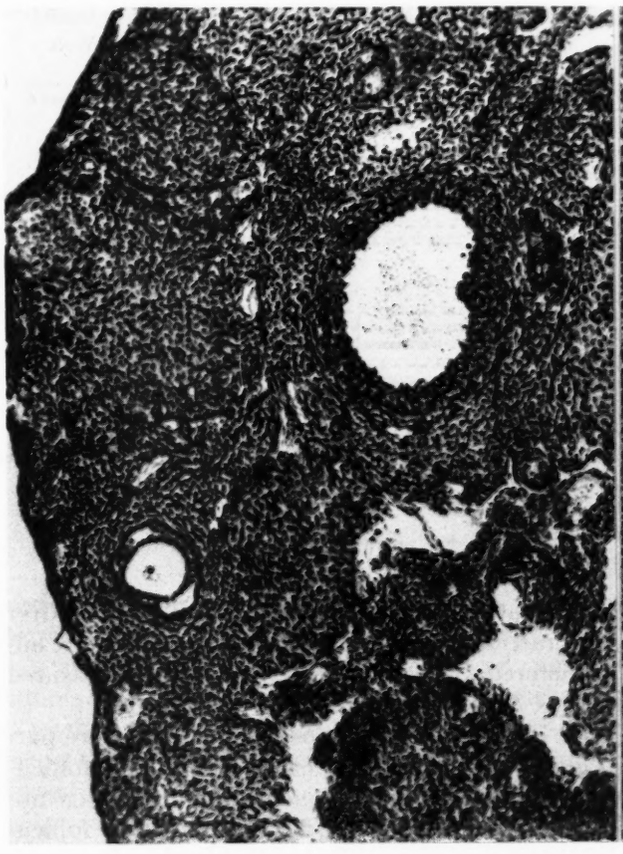

FIG. 3.—Photomicrograph of a large part of an intrasplenic ovarian graft that had persisted 246 days in a unilaterally ovariectomized mouse. Several small and medium-sized follicles are present. ×105.

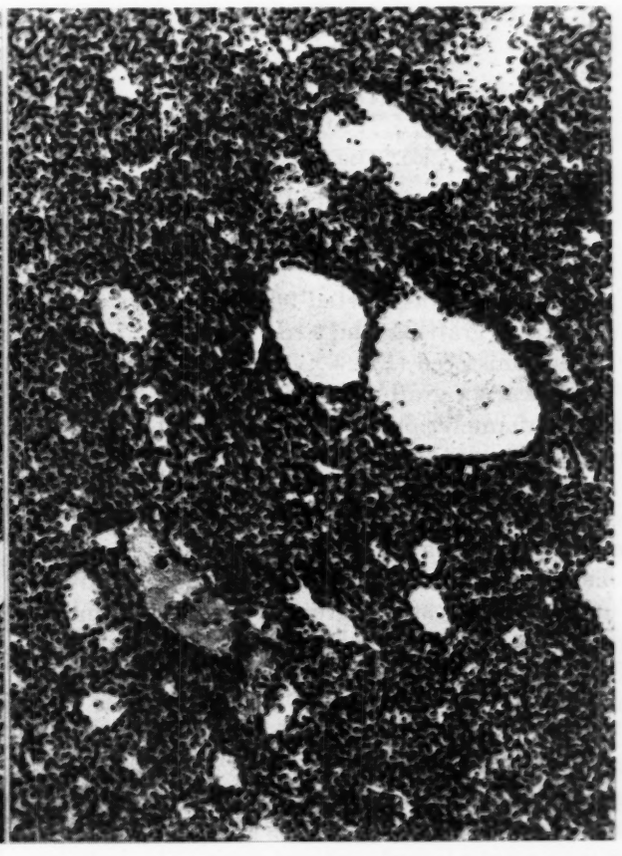

FIG. 4.—A small area of a large granulosa-cell tumor that had arisen in an intrasplenic ovarian graft in an ovariectomized mouse. No follicles were present in this graft. ×105.

sive changes were observed in the two other grafts, although they contained ovarian stroma and few primary follicles.

The urinary bladders were also distended in the female hosts. Occasional cornified vaginal smears were obtained from one female mouse before estrogen treatment was started. The average uterine weight was 149 mg. The histological appearance of the adrenal glands resembled that of the males.

D. Ovarian Grafts in Androgen-treated Castrated Mice

Castrated males.—No tumors developed in intrasplenic ovarian grafts in 8 castrated male mice that had been treated with testosterone propionate.

Gonadectomized Mice with Intrasplenic Ovarian Grafts
16 μgm. α-Estradiol Benzoate Injected per Week

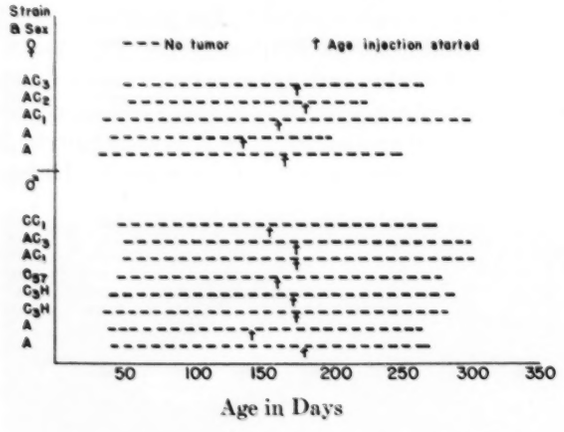

Fig. 5.—For meaning of symbols refer to Fig. 1

The treatment was started 96 to 130 days subsequent to the grafting, and 9 to 17 weekly injections were administered (Fig. 6).

The ovarian grafts ranged from 2 to 6 mm. in greatest diameter. A unique histological feature of these grafts was the increase of fibrous connective tissue in, and vascularity of, the stroma. With the exception of two degenerate grafts, they consisted largely of ovarian follicles and a few atretic and hemorrhagic follicles. No corpora lutea were noted. Tubular and cystic ingrowths of the germinal epithelium were observed in three grafts, and most of these structures contained coagulated fluid and macrophages.

The seminal vesicles and prostates of the androgen-treated male hosts were large, and their average weight was 0.35 gm. No x-zones were noted in the adrenal glands, and groups of small subcapsular cells were present in some areas. The kidneys and submaxillary glands were of the male type.

Castrated females.—Six castrated female mice, bearing intrasplenic ovarian grafts, received 13 to 17 weekly injections of testosterone propionate, starting 101 to 128 days subsequent to the grafting. The ovarian grafts were small and nontumorous (Fig. 6). Their histological structures were similar to those in the male hosts, although more luteinized interstitial cells were present in the stroma. In two grafts, germinal epithelial cells formed papillary ingrowths. Large cysts or tubular cavities were derived from ingrowths of the same type of cell. Extensive degeneration was observed in one of the six grafts.

An estrous vaginal smear was obtained in one female host before the injection of testosterone propionate was started. The average uterine weight of this group was 133 mg. Other organs exhibited evidence of androgenic effects, as in the male hosts.

E. Ovarian Tumors in Progesterone-treated Castrated Mice

Castrated males.—Five granulosa-cell tumors and two mixed tumors—both granulosa and luteoma cells—arose in the intrasplenic ovarian grafts in 8 castrated males treated with progesterone.

Gonadectomized Mice with Intrasplenic Ovarian Grafts
1.25 mg. Testosterone Propionate Injected per Week

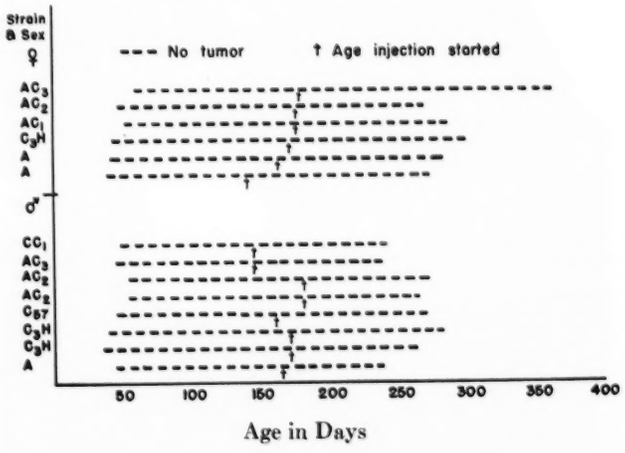

Fig. 6.—For meaning of symbols refer to Fig. 1

The treatment was started 115 to 138 days after the grafting, and 9 to 19 weekly injections were administered (Fig. 7). The largest tumor measured 14.5 × 15 × 21 mm.

In general, the granulosa-cell tumors were partially encapsulated and exhibited diffuse, folliculoid, or trabecular arrangements. Numerous mitotic figures indicated rapid growth. Some folliculoid structures were filled with blood, others with coagulated material containing a few degenerating cells. Luteinized interstitial cells were noted adjacent to small edematous or necrotic areas. Prolifer-

ation and tubular ingrowths of the germinal epithelium were present in four of these tumors. A spicule of bone developed at the periphery of the largest granulosa-cell tumor, and the tumor had metastasized to the liver. This tumor was transplanted subcutaneously into other mice of the same strain (6).

The mixed tumors were composed of masses of granulosa and luteoma cells, in addition to areas of luteinized interstitial cells and ingrowths of the germinal epithelium. The one nontumorous graft was 139 days old and consisted primarily of luteinized interstitial cell stroma and ovarian follicles. Some of the follicles were filled with blood. Four other intrasplenic ovarian grafts with vascularized adhesions were not included in this paper.

The seminal vesicles and prostates of the male hosts were atrophic. The submaxillary glands and the kidneys resembled those of castrated animals.

Castrated females.—Three granulosa-cell tumors, one luteoma, and one mixed tumor developed in intrasplenic ovarian grafts in 5 castrated females treated with progesterone. The treatment was started 127 to 134 days after the grafting; 14 to 19

Gonadectomized Mice with Intrasplenic Ovarian Grafts
1 mg. Progesterone Injected per Week

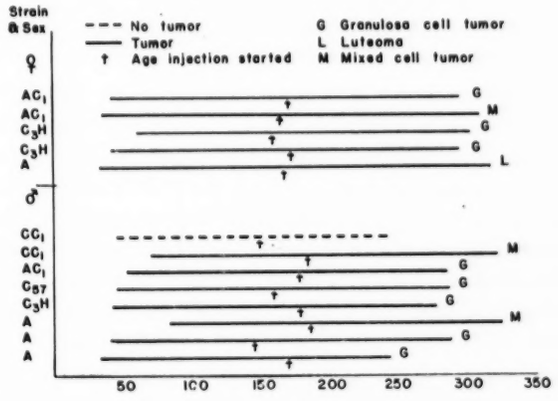

FIG. 7.—For meaning of symbols refer to Fig. 1

weekly injections were administered (Fig. 7). The largest tumor measured 12 × 15 × 17 mm.

Histologically, the granulosa-cell and mixed tumors resembled those in the male hosts, with the exception of more extensive areas of luteinized tissue and necrosis. No metastasis was noted from these tumors.

An estrous vaginal smear was observed once, before the progesterone injections were started, in a female mouse of the C_3H strain that had the largest granulosa-cell tumor, whose uterus weighed 150 mg. at autopsy. The average uterine weight of the other females was 41 mg. Hyperplasia of the small subcapsular cells was noted in three adrenal glands.

F. Ovarian Tumors in Gonadotrophin-treated Castrated Mice

Castrated males.—One granulosa-cell tumor and two mixed tumors were found in intrasplenic ovarian grafts in 5 castrated males treated with daily injections of PMS, starting 100 to 119 days after the grafting (Fig. 8). The largest tumor measured 4 × 4 × 6.5 mm. (Fig. 9). One nontumorous ovarian graft was attached to the peritoneum of the body wall and the other to the small intestines.

The seminal vesicles and prostates of the male hosts were atrophic. The adrenals showed degeneration of the x-zones. The histological structure of

Gonadectomized Mice with Intrasplenic Ovarian Grafts
25 I.U. Gonadotrophin (PMS) Injected per Day

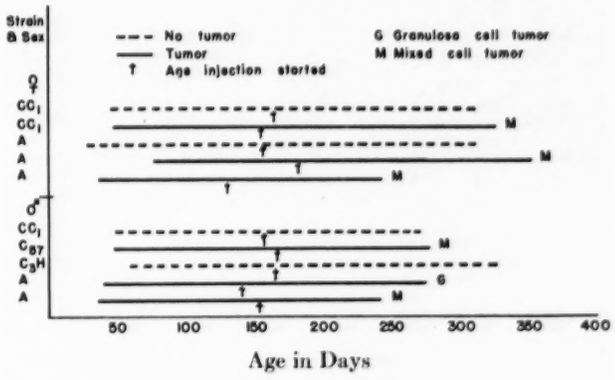

FIG. 8.—For meaning of symbols refer to Fig. 1

the kidneys and submaxillary glands was similar to that of the gonadectomized male mice.

Castrated females.—Three mixed tumors developed in intrasplenic ovarian grafts in 5 castrated females treated with daily injections of PMS, starting 92 to 127 days after the grafting (Fig. 8). The largest tumor measured 5 × 7 × 8 mm.

The histological appearance of the mixed tumors was similar to that of the male hosts similarly treated. In one tumor, spicules of bone were observed in a necrotic area adjacent to the granulosa-tumor cells. Numerous osteoblasts lined the surfaces of the bone matrix, and fibroblasts and macrophages were scattered around the osseous tissue. The two nontumorous grafts were small and consisted of a few ovarian follicles, corpora lutea, and degenerative connective tissue stroma.

Irregular estrous vaginal smears were obtained in one female before the daily injections of PMS were started; no cornified estrous smears were noted in the female hosts following treatment. The

average uterine weight was 42 mg. The x-zone of the adrenal glands was absent. As in the castrated males, the parietal layer of the renal corpuscles were usually composed of low epithelial cells. The terminal tubules of the submaxillary glands in two animals bearing tumorous grafts, however, consisted of columnar cells.

DISCUSSION

The present experiments provide further evidence that pituitary gonadotrophic hormones are involved in ovarian tumorigenesis, at least in the experimental ovarian tumorigenesis in castrated mice bearing intrasplenic ovarian transplants. The failure of ovarian tumors to appear in the intrasplenic ovarian grafts in unilaterally castrated male and female mice shows that the intact gonads inhibit tumor formation in the transplants. These observations support those made previously that intrasplenic grafts with extensive adhesions to the body wall or uterus—areas drained by other than the hepatic portal system—did not become tumorous (6, 7). Both the intact gonad as well as the transplanted gonads, if connected with the caval venous system, inhibit tumorigenesis.

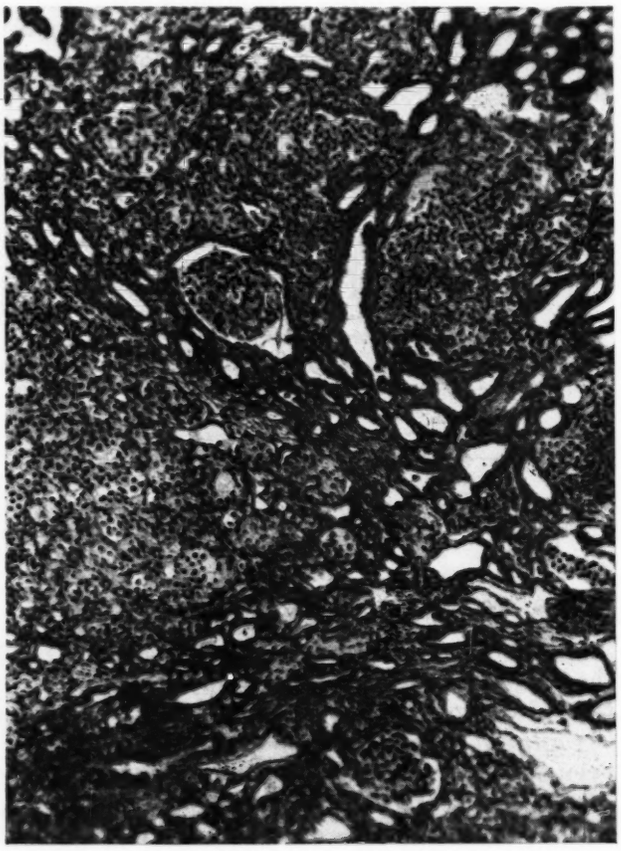

FIG. 9.—A small section of a tumorous graft that had been in the spleen of a castrate male mouse for 268 days. This mouse had received gonadotrophin (pregnant mare's serum). The area of the graft photographed shows epithelial-lined spaces surrounded by partially luteinized cells. The epithelial cords are continuous with the germinal epithelium that partially surrounds all well-developed grafts. Some of the tubules contain cells similar to those in the intertubular areas. ×105.

Whether the steroid hormones usually presumed to be produced by the gonads—namely, estradiol and testosterone—or some other or unknown substances inhibited tumorigenesis of the transplants was not revealed by experiments of the type referred to above. The failure of ovarian tumors to appear in intrasplenic ovarian grafts in castrated mice that were given injections of estradiol benzoate or testosterone propionate indicates that the endocrine products presumably produced by the normal gonads inhibit the formation of granulosa-cell tumors and luteomas. Progesterone in the amounts used and with the weekly schedule of injection did not alter the incidence of tumors. Larger amounts might be effective. Burrows and Hoch-Ligeti (3) failed to alter the incidence of mammary tumors in mice of the C_3H strain given weekly doses of 1 mg. of progesterone.

The observations on the group of mice that received the gonadotrophic preparation (PMS—Anteron) were inconclusive. Such preparations produce a marked luteinization of the ovaries (12, 14), and the ovaries so treated produce substances that have an androgenic effect (11, 12). At the present time it is assumed that the pituitary stimulation responsible for tumorigenesis in intrasplenic grafts in castrated mice is primarily the follicle-stimulating hormone. It is well known that the urine of postmenopausal women contains increased amounts of a substance that is primarily follicle-stimulating. The occurrence of mixed granulosa-cell tumors and luteomas in the castrate male mice indicates that the gonadotrophin did have some effect, as such tumors are noted rarely among untreated, graft-bearing male mice.

Whether or not the tumors arising in intrasplenic transplants are comparable to those arising in x-rayed mice has been questioned (4). Certainly, they possess a capacity to metastasize to the liver (4, 6), and several of them have been transplanted successfully into other and untreated hosts of the same strains. Intrasplenic ovarian grafts when irradiated did not become tumorous more rapidly than when they were not irradiated (10). The period subsequent to irradiation at which tumors appear is longer than that subsequent to transplantation into the spleens of castrate mice. If the theory that ovarian tumors are induced in irradiated mice by attainment of a hormonal imbalance proves to be correct, then the hormonal factors attained sub-

sequent to irradiation must be less favorable. Irradiated ovaries do produce periodic vaginal cornification in mice; Brambell, Parkes, and Fielding (2) described estrous cycles in mice made anovular by roentgen irradiation. A low level of estrogen production may reduce the increased production of gonadotrophin and hence increase the length of the period of stimulation required.

Unlike tumors of some types, the ovarian tumors appear in mice of all strains that have been studied up to this time. Similar tumors were originally described in rats (1).

The tumors produce estrogen, as the uteri of the hosts bearing them are larger than those of castrated animals. The estrogen must be produced in amounts sufficiently large to permit some of it to pass through the liver or to be of a type not inactivated by the liver. Several animals showed hepatic damage, and one showing the most extensive indications of damage had a large uterus weighing 135 mg.

Adrenal tumors have been described in mice castrated at birth (15, 16) or at later ages (5). Strain differences in the tendency for such tumors to appear have also been described (13, 16). None of the mice in the present study had such tumors. The period of survival was not sufficiently long for them to have been expected. They have been noted, however, in x-irradiated mice (unpublished).

SUMMARY AND CONCLUSIONS

1. Granulosa-cell tumors, luteomas, and mixed tumors (granulosa and luteoma cells) developed in intrasplenic ovarian grafts in castrated male and female mice. There is apparently no strain limitation in the formation of these tumors in mice.

2. No ovarian tumors were observed in the intrasplenic grafts of ovaries in (*a*) unilaterally gonadectomized mice, (*b*) gonadectomized mice that received estradiol benzoate or testosterone propionate, or (*c*) gonadectomized mice with vascularized adhesion that permitted ovarian hormones to by-pass the hepatic portal circulation. The gonadal hormones are assumed to act indirectly by inhibiting the production and secretion of the pituitary gonadotrophic hormones.

3. Weekly treatment with 1 mg. of progesterone did not prevent tumor formation in intrasplenic ovarian grafts.

4. Daily injections of a gonadotrophin from the pregnant mare's serum (PMS) exerted luteinizing influence on the ovarian tumors.

5. The malignancy of the induced granulosa-cell tumors is shown by the ability to metastasize and the transplantability into new hosts.

6. These experimental results appear to substantiate further the assumption that prolonged stimulation by increased amounts of gonadotrophic hormones is responsible for the genesis of ovarian tumors.

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Dehydrogenase Studies of Tissue from Normal and Tumor-bearing Mice
I. Total Dehydrogenase Activity

SR. M. AGATHA RIEHL, O.S.B., AND SR. M. PETRA LENTA, O.S.B.

(From the College of St. Scholastica, Unit of the Institutum Divi Thomae, Duluth 2, Minnesota)

Evidence has been accumulated that several components of enzyme systems important in biological oxidations are altered or impaired in malignancy. While numerous reports have appeared on the dehydrogenases in normal and neoplastic tissues, many phases of the problem remain uninvestigated, and much of the earlier work needs confirmation and supplementation.

It would seem that the deficiencies reported in the various oxidative systems of tumor tissues—for example, cytochrome c and cytochrome oxidase (3, 10, 12)—would result in an accumulation of reducing substances in the tissue and that the altered oxidative mechanism would be associated with changes in the individual enzyme systems. These considerations led to the investigation of (*a*) the comparative ability of various tissues of normal and tumor-bearing animals to decolorize methylene blue anaerobically, the results of which are presented herewith, and (*b*) the comparative activity of the specific dehydrogenases of the various organs of normal and tumor-bearing animals, the results of which are presented in a subsequent report.

A decreased ability of certain types of tumor tissue to decolorize methylene blue has been reported (6, 7), but Barron did not observe such a difference (1). Reports of the influence of the presence of a tumor on the enzymatic activities of organs remote from the site of the tumor (4, 5) prompted the analysis of the dehydrogenase activity of a number of different organs in tumor-bearing animals. Furthermore, it seemed desirable to determine whether differences in susceptibility of various strains of mice to transplanted tumors were correlated with alterations in the dehydrogenase activity.

Schlenk and others (11, 2, 8) have reported that there is a reduction in the content of coenzyme I in tumor tissues. Also, as Von Euler (12) and others have shown, coenzyme I in malignant tissues is present mainly in the dihydro form, whereas in most normal tissues it exists chiefly in the oxidized state. Since this important biocatalyst is so definitely altered in neoplastic tissues, it was of interest to investigate the activity of dehydrogenase systems of normal and tumor tissues when coenzyme I was added in varying concentrations to dehydrogenase-containing extracts of various organs of normal and tumor-bearing animals.

EXPERIMENTAL

Mice of widely varying ages and both sexes of the homozygous dba (38 animals) and C57 (25 animals) strains and the heterozygous Rockland albino (28 animals) strain were used. All animals were maintained on a standard Rockland mouse-pellet diet. The tumors studied included the transplanted dbrB adenocarcinoma and sarcoma 180, as well as methylcholanthrene-induced tumors. At the time of experiment the tumors ranged between 8 and 25 mm. in diameter.

The animals were killed by decapitation, and the organs to be analyzed (i.e., skeletal muscle from the hind limbs and the spleen, liver, kidney, and brain) were immediately removed. These organs were weighed on an analytical balance and then ground with washed and ignited sand in a mortar with a small volume of cold distilled water to the consistency of a smooth thin paste, 2 ml. of water being used for each 0.1 gm. (wet weight) of tissue. Care was taken to rinse the mortar thoroughly with the last few milliliters of the calculated volume of cold water. These washings were then added to the extracted mixture, which was placed in a centrifuge tube, stirred well, and then centrifuged at 800 r.p.m. for 5 minutes. The centrifugate was removed and tested immediately for its ability to decolorize methylene blue anaerobically according to the Thunberg technic.

In measuring the dehydrogenase activity, 2 ml. of chilled tissue extract were placed in the stopper of the Thunberg tube. Into the tube proper the following reagents were introduced: 0.25 ml. of a 1:5000 methylene blue solution and 1.0 ml. of phosphate buffer, pH 7.2. The tubes were evacu-

ated with gentle agitation for 5 minutes at about 12 mm., after which the contents were brought to 37° C. in a constant-temperature water bath by allowing an equilibrium period of 10 minutes. The enzyme-containing tissue extract was then tilted into the methylene blue–buffer mixture and the decolorization time determined visually. Each test was made in duplicate. All extracts and organs were kept at 0° C. when not in use. Necrotic and hemorrhagic portions of the tumors were carefully excised and discarded before analysis. The enzyme activity was arbitrarily expressed as minutes$^{-1} \times 10^2$. With proper precautions the results were readily reproducible.

properly speaking, this is a measure of the total reducing substances present in the extract of the tissue. In calling such a reaction the "total dehydrogenase activity," it was presumed that the crude tissue extracts prepared in the manner described contained sufficient quantities of the substrates, since the addition of several substrates operating in systems requiring coenzyme I did not cause significant changes in the decolorization time by the crude extracts. In a number of cases, however, the addition of substrates not requiring coenzyme I speeded up the activity to some extent.

The reliability of the technic was shown by the fact that in 96 per cent of the duplicate or triplicate

TABLE 1

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF SKELETAL MUSCLE FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	COENZYME I (MG.)	NO. DETERMINATIONS	DECOLORIZATION TIME (MIN.) Mean	Range	S.D.	VELOCITY (MIN.$^{-1} \times 10^2$)	PER CENT STIMULATION BY COENZYME
NORMAL ANIMALS—MUSCLE							
dba	0.0	14	28.7	(20.0–35.0)	±4.46	3.5	457
	0.5	12	5.1	(2.5– 7.0)	±1.64	19.5	
Rockland	0.0	18	30.2	(21.0–36.0)	±4.65	3.3	1021
	0.5	14	2.7	(2.0– 4.0)	±0.75	37.0	
C57	0.0	22	28.3	(20.0–34.0)	±4.73	3.5	765
	0.5	18	3.3	(2.5– 4.0)	±0.48	30.3	
TUMOR-BEARING ANIMALS—MUSCLE							
dbrB in dba	0.0	18	28.5	(21.0–32.0)	±3.86	3.5	548
	0.5	18	4.4	(2.5– 6.0)	±1.53	22.7	
Methylcholanthrene in dba	0.0	4	25.0	(20.0–30.0)	±5.00	4.0	732
	0.5	4	3.0	(3.0)	0.00	33.3	
S 180 in Rockland	0.0	16	27.6	(20.0–34.0)	±5.83	3.6	891
	0.5	12	2.8	(2.0– 3.5)	±0.62	35.7	

Coenzyme I was prepared according to the method of Williamson and Green (13). While the degree of purity of the product was not determined experimentally, comparable results could be obtained by standardizing one preparation against the next. A suitable concentration was selected for investigation of its comparative effects on the dehydrogenase activity of extracts of tissues from normal and tumor-bearing animals by adding varying amounts of the coenzyme I to the extracts of tissues from normal dba mice. The tissue extracts were so diluted that a definite alteration of dehydrogenase activity was apparent when the amount of coenzyme I was varied as determined in preliminary experiments. The same type of study was made with extracts of the adenocarcinoma dbrB and sarcoma 180.

The ability of a tissue extract to reduce methylene blue in an oxygen-free medium without the addition of substrates or coenzymes was designated as the "total dehydrogenase activity." More

determinations the total dehydrogenase activity varied by not more than 2 minutes, and the variations for organs from animals of the same strain did not exceed ±3 minutes. The results presented in this paper represent 840 determinations.

RESULTS

There was a marked similarity of the total dehydrogenase activity of analogous organs of normal animals of different strains (Tables 1–5). The presence of the transplanted tumor did not significantly alter this activity in any of the organs studied. The order of increasing dehydrogenase activity in both normal and tumor-bearing mice of all strains was as follows: muscle, spleen, brain, kidney, and liver. Furthermore, a difference in susceptibility to the dbrB tumor in the C57 and dba strains was not reflected in the values for the total dehydrogenase activity.

There was apparently a distinct difference be-

tween dehydrogenase activity of the methylcholanthrene-induced tumor and sarcoma 180, on the one hand, and the dbrB adenocarcinoma, on the other (Table 6), although relatively few methylcholanthrene-induced tumors were available for study. This difference may have been due in part to the fact that the calculations were made on the basis of the wet weight; by dry-weight determinations the adenocarcinoma contained 85 per cent water, as compared to the sarcoma, which contained 72 per cent water.

The addition of coenzyme I accelerated the decolorization time of methylene blue by spleen extracts much less than it did the other normal tissues (Tables 1–5), and this was true also in additional experiments in which coenzyme I was added in amounts up to 1.5 mg. In unreported experiments both the adenocarcinoma and the sarcoma 180 responded markedly to the addition of coenzyme I in varying concentrations. Results from the addition of coenzyme I at a level of 0.5 mg. to extracts of tumor tissue are shown in Table 6. The percentage stimulation of dehydrogenase activity was higher than that in similar preparations of spleen (Table 2) but was lower than that of liver (Table 5), kidney (Table 4), brain (Table 3), and muscle (Table 1).

DISCUSSION

Perhaps the most significant observation to be made in these studies is the marked similarity and stability of the activity of the so-called "total" dehydrogenases. As shown by the experimental data, this ability to decolorize methylene blue was independent of animal age, sex, and strain. Furthermore, the presence of a transplanted tumor (sar-

TABLE 2

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF SPLEEN FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

Strain of mice	Coenzyme I (mg.)	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}$ × 10^2)	Per cent stimulation by coenzyme
Normal animals—spleen							
dba	0.0	10	10.8	(9.0–13.0)	1.47	9.2	44
	0.5	8	7.5	(7.0– 9.0)	±0.86	13.3	
Rockland	0.0	18	12.1	(9.0–15.0)	±1.79	8.2	22
	0.5	10	10.0	(7.0–12.0)	±1.69	10.0	
C57	0.0	8	11.8	(10.0–14.0)	±1.48	8.4	32
	0.5	8	9.0	(8.0–11.0)	±1.23	11.1	
Tumor-bearing animals—spleen							
dbrB in dba	0.0	22	12.0	(8.0–18.0)	±3.17	8.3	28
	0.5	16	9.3	(6.0–14.0)	±3.56	10.7	
Methylcholanthrene in dba	0.0	6	12.6	(8.0–13.0)	±2.69	7.8	60
	0.5	6	8.0	(6.0–11.0)	±2.24	12.5	
S 180 in Rockland	0.0	12	10.0	(8.0–11.0)	±1.15	10.0	25
	0.5	12	8.0	(7.0–10.0)	±0.93	12.5	

TABLE 3

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF BRAIN FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

Strain of mice	Coenzyme I (mg.)	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}$ × 10^2)	Per cent stimulation by coenzyme
Normal animals—brain							
dba	0.0	8	8.5	(6.0–11.0)	±1.80	11.7	327
	0.5	8	2.0	(1.5– 2.5)	±0.35	50.0	
Rockland	0.0	16	9.8	(8.0–13.0)	±1.61	10.2	307
	0.5	16	2.4	(2.0– 3.0)	±0.48	41.6	
C57	0.0	8	10.5	(10.0–11.0)	±0.50	9.5	356
	0.5	8	2.3	(2.0– 3.0)	±0.37	43.4	
Tumor-bearing animals—brain							
dbrB in dba	0.0	14	8.1	(6.0–13.0)	±2.35	12.3	199
	0.5	14	2.2	(2.0– 4.0)	±0.70	36.8	
Methylcholanthrene in dba	0.0	4	10.0	(10.0)	0.00	10.0	300
	0.5	4	2.5	(2.0– 3.0)	±0.50	40.0	
S 180 in Rockland	0.0	6	9.3	(8.0–10.0)	±0.94	10.7	305
	0.5	6	2.3	(2.0– 3.0)	±0.47	43.4	

TABLE 4

Decolorization Time of Methylene Blue by Aqueous Extracts of Kidney from Normal and Tumor-bearing Mice in the Presence and Absence of Coenzyme I

Strain of mice	Coenzyme I (mg.)	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}\times10^2$)	Per cent stimulation by coenzyme
Normal animals—kidney							
dba	0.0	12	4.40	(4.0 –5.0)	±0.45	22.7	267
	0.5	10	1.20	(0.5 –1.5)	±0.40	83.3	
Rockland	0.0	12	2.60	(2.0 –3.5)	±0.68	38.4	391
	0.5	12	0.53	(0.4 –0.7)	±0.10	188.6	
C57	0.0	30	4.20	(3.0 –6.0)	±0.89	23.8	250
	0.5	18	1.20	(0.7 –2.0)	±0.37	83.3	
Tumor-bearing animals—kidney							
dbrB in dba	0.0	24	3.20	(2.0 –6.0)	±1.08	31.2	237
	.5	24	0.95	(0.4 –2.0)	±0.48	105.2	
Methylcholanthrene in dba	0.0	4	2.40	(2.25–2.5)	±0.17	41.6	380
	0.5	4	0.50	(0.3 –0.7)	±0.63	200.0	
S 180 in Rockland	0.0	10	2.50	(2.0 –3.0)	±0.45	40.0	245
	0.5	10	0.73	(0.7– 0.75)	±0.02	138.0	

TABLE 5

Decolorization Time of Methylene Blue by Aqueous Extracts of Liver from Normal and Tumor-bearing Mice in the Presence and Absence of Coenzyme I

Strain of mice	Coenzyme I (mg.)	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}\times10^2$)	Per cent stimulation by coenzyme
Normal animals—liver							
dba	0.0	16	1.80	(1.5–2.0)	±0.24	55.5	350
	0.5	10	0.40	(0.3–0.5)	±0.10	250.0	
Rockland	0.0	14	1.30	(1.1–2.0)	±0.30	76.1	245
	0.5	14	0.38	(0.3–0.5)	±0.07	263.0	
C57	0.0	30	1.70	(1.1–3.0)	±0.31	58.8	226
	0.5	16	0.52	(0.3–1.0)	±0.20	192.1	
Tumor-bearing animals—liver							
dbrB	0.0	24	1.80	(1.0–2.0)	±0.36	55.5	363
	0.5	10	0.40	(0.3–0.5)	±0.09	250.0	
Methylcholanthrene in dba	0.0	4	1.30	(1.3–1.3)	0.00	76.1	228
	0.5	4	0.40	(0.3–0.5)	±0.10	250.0	
S 180 in Rockland	0.0	10	1.30	(1.0–1.7)	±0.22	76.1	245
	0.5	10	0.38	(0.3–0.5)	±0.08	263.0	

TABLE 6

Decoeorization Time of Methylene Blue by Aqueous Extracts of Tumors in the Presence and Absence of Coenzyme I

Strain of mice	Tumor type	Coenzyme I (mg.)	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}\times10^2$)	Per cent stimulation by coenzyme
dba	dbrB adenocarcinoma	0.0	20	16.8	(14.0 –22.0)	±2.18	5.9	181
		0.5	14	6.0	(5.0 – 7.0)	±0.84	16.6	
dba	Methylcholanthrene	0.0	4	4.1	(3.3 – 5.0)	±0.85	24.4	156
		0.5	4	1.6	(1.25– 2.0)	±0.40	62.5	
Rockland	Sarcoma 180	0.0	24	5.4	(4.0 – 6.5)	±0.76	18.5	116
		0.5	24	2.5	(2.0 – 3.0)	±0.32	40.0	

coma or carcinoma) caused no change in the activity of the animal tissues. In contrast to the reports of deficiencies and changes in the oxidative metabolism of tumor tissue, suggesting an accumulation of reducing substances, the results of the present study indicate no significant increase in the amount of water-soluble materials capable of reducing methylene blue in the organs of tumor-bearing animals. Strictly speaking, comparison of the activity of tumor tissue with any except that of the analogous normal tissue is unwarranted; but it can be observed that this activity in tumor tissue showed no striking difference from that of certain nonneoplastic tissues. Tumor tissue appeared to have an activity rate intermediate between that of the more active normal liver and kidney tissue extracts, on the one hand, and the less active extracts of brain, spleen, and muscle tissue, on the other. Whether the tumor causes alterations in specific dehydrogenase systems of organs remote from the site of the neoplasm is to be the subject of future studies, but it is probable that such variations would not be detected by the methods used in the present study.

The results obtained when coenzyme I was added in varying concentrations to tumor extracts would not suggest that reducing substances were present in large enough quantities or in a sufficiently potent state to inhibit the coenzyme activity of systems containing concentrations of coenzyme I greater than those normally present in the tissue.

The failure of large amounts of coenzyme I to effect a significant stimulation of dehydrogenase activity of the spleen in both normal and tumor-bearing animals cannot be adequately interpreted at present. According to the report of Bernheim and Felsovanyi (2), the coenzyme content of spleen is slightly higher than that of liver, kidney, and muscle. If coenzyme I is normally present in excess in the spleen, additional amounts of coenzyme I would hardly result in an acceleration of activity. The possibility is not precluded, however, that certain coenzyme I–requiring dehydrogenases are present in diminished quantities or are entirely lacking. As indicated in this study, spleen extract is not the least active of extracts of normal tissues in decolorizing methylene blue, but subsequent investigations of individual enzyme systems (9) have revealed slight or no lactic dehydrogenase activity in the spleen. The malic dehydrogenase content of the spleen is also quite low, having about the same decolorization time as malic dehydrogenase in the several different tumors analyzed.

SUMMARY

Quantitative determinations of the total dehydrogenase activities of the muscle, liver, kidney, brain, and spleen of normal homozygous dba and C57 mice and heterozygous Rockland albino mice revealed a remarkable similarity of activity among the various animal strains. The presence of a tumor (transplanted dbrB adenocarcinoma, methylcholanthrene-induced tumors in dba mice, and transplanted sarcoma 180 in Rockland mice) did not alter the ability of any of the tissue extracts studied to decolorize methylene blue. The total dehydrogenase activity was independent of the sex, strain, and age of the animals, both normal and tumor-bearing. The addition of 0.5 mg. of coenzyme I markedly accelerated the total dehydrogenase activity of the muscle, liver, and brain of normal and tumor-bearing animals, the degree of acceleration in tissues from tumor-bearing animals being about the same as that in tissues from normal animals. The addition of the same amount of coenzyme I to extracts of the different tumors resulted in a pronounced stimulation of activity. The total dehydrogenase activity of the spleen from normal and tumor-bearing animals was not accelerated to any appreciable extent by the addition of a large amount of coenzyme I.

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Dehydrogenase Studies of Tissue from Normal and Tumor-bearing Mice
II. Lactic and Malic Dehydrogenases

SR. M. PETRA LENTA, O.S.B., AND SR. M. AGATHA RIEHL, O.S.B.

(From the College of St. Scholastica, Unit of the Institutum Divi Thomae, Duluth 2, Minnesota)

A high concentration of lactic acid in rat hepatoma has been reported by Kishi, Fujiwara, and Nakahara (5), as compared with the content of normal liver. Undoubtedly, this accumulation of lactic acid in tumor tissues is associated with the high rate of glycolysis in malignant tissues; but, since so little lactate is removed by oxidative processes, an impairment of enzyme activity is suggested. Elliott, Benoy, and Baker (1) have reported that the ability of tumor tissue to oxidize lactate to pyruvate, and succinate to fumarate, appeared to be defective. These investigators also found that the formation of succinate from pyruvate seemed to proceed at a fair rate and that an equilibrium between fumarate and malate could occur in tumor tissue. Waterman (11) concluded from his experiments that lactic dehydrogenase was lacking in cancerous tissue; but Von Euler, Malmberg, and Günther (9) have reported that a dialyzed extract of Jensen rat sarcoma rapidly oxidized lactic and malic acids. Von Euler, Adler, and Günther (8) further observed that the lactic and malic dehydrogenase systems of the Jensen rat sarcoma were not appreciably different from those of muscle. Von Euler, Malmberg, Günther, and Nystrom (10), however, considered that, while lactic acid and other hydrogen donators were present in excess in Jensen sarcoma, the deficient oxidative mechanism was associated with a lowered activity of the flavin enzymes and coenzymes. More recently, Potter (6) has investigated the malic dehydrogenase activity of normal rat liver and rat hepatoma. In the complete malic dehydrogenase system, the oxygen uptake of hepatoma was found to be much lower than that of normal liver.

The present study was designed to analyze the comparative lactic and malic dehydrogenase activities of various organs of normal and tumor-bearing mice.

EXPERIMENTAL

Of the animals used, 8 were normal C57, 35 were dba, and 31 were Rockland albino mice of both sexes and different ages. The dbrB adenocarcinoma was carried in dba mice, and the sarcoma 180 in the Rockland mice; at the time of experiment the tumors ranged between 8 and 25 mm. in diameter. All animals were maintained on a standard Rockland mouse-pellet diet. In preparing the tumor tissue for analysis, the necrotic and hemorrhagic areas were carefully removed.

The lactic and malic dehydrogenases were prepared according to the method of Green (2) with modifications. Essentially, the procedure consisted in the preparation of a dialyzed extract of enzymes obtained from the tissues as an acetone powder. According to the method of Green (2), the original tissue was allowed to extract for ½ hour with ice water before precipitation with acetone. In the present study this extraction period was omitted because the amount of tissue rarely exceeded 1.5 gm. wet weight. The entire mixture was precipitated with acetone without previously pressing it through muslin, as prescribed by Green's method.

Immediately upon decapitation of the animals the organs to be analyzed were weighed and then ground thoroughly with a very small quantity of purified sand and a few milliliters of cold distilled water. The resulting thin paste was then poured into 3 volumes of cold acetone. The precipitate thus formed was filtered on a very small Büchner funnel and then washed with acetone and ethyl ether. This dried acetone powder was freed of the last traces of ether and then rubbed up with a small volume of cold distilled water, 2.0 ml. of water being used for every 0.1 gm. (wet weight) of tissue. The suspension was transferred quantitatively to Cellophane bags and dialyzed overnight against distilled water at 0° C. The preparation was then centrifuged at 800 r.p.m. for about 5 min-

utes and the precipitate discarded. The supernatant, which contained both lactic and malic dehydrogenase, was removed immediately and kept at 0° C. when not in use.

The dehydrogenase activity of the tissue preparations was measured according to the Thunberg technic. To the Thunberg tube proper were added 0.25 ml. methylene blue 1:5000; 1.0 ml. phosphate buffer, pH 7.2; 0.5 ml. of a 0.1 *M* substrate (sodium lactate or sodium malate); 0.2 ml. of a 2 *M* potassium cyanide adjusted to pH 8.0; and 0.5 mg. of a coenzyme I solution having a concentration of about 1 mg. per milliliter. Two ml. of the chilled enzyme solution were placed in the hollow stopper of the Thunberg tube.

Coenzyme I was prepared according to the method of Williamson and Green (12). Although the percentage purity was not determined, successive batches were standardized against one another. The strong potassium cyanide solution was added to fix the products formed in the dehydrogenase reaction. The evacuation of the tubes was accomplished with a high-vacuum pump at about 12 mm. for 5 minutes and was facilitated by gentle agitation of the tubes during this time. The tubes were then placed in a constant-temperature water bath at 37° C. for 10 minutes. At the end of this equilibration period the enzyme-containing solution was allowed to react with the substrate, and the time required for decolorization of the methylene blue was determined. Tests were made in duplicate. The dehydrogenase activity was expressed at $\text{minutes}^{-1} \times 10^2$.

RESULTS

The values obtained for the malic and lactic dehydrogenases in various organs from normal and tumor-bearing mice, as well as in the dbrB adenocarcinoma and sarcoma 180 transplanted tumors are given in Tables 1–6, in which the results of 924 determinations are reported. Eighty-nine per cent of the duplicate determinations checked within 2 minutes, the remainder varying not more than 4 minutes. There was a variation of ± 3 minutes in the enzyme activity of analogous organs from animals of the same strain. The rate of decolorization in the presence of tumor tissue varied within a maximum of ± 5 minutes, with two-thirds varying less than ± 2 minutes.

In the case of the lactic dehydrogenase activity of the spleen of both normal and tumor-bearing animals, complete decolorization in 72 per cent of the 33 determinations did not take place within 2 to 3 hours. There was, however, a change from the original deep-blue color to a green within the first half-hour of that time, but further decolorization was not appreciable after that period. In the remaining 9 instances (28 per cent), complete decolorization occurred within 20 to 60 minutes. This variation in activity may be associated with the hemopoietic nature of the organ and the amount of blood present.

DISCUSSION

While the presence of tumors has been associated with the lowering of certain enzyme activities in organs remote from the site of the neoplasm and not invaded by the tumor (4), in the experiments reported herein neither the lactic nor the malic dehydrogenase activity of organs of animals bearing transplanted tumors appears to be influenced by the growing neoplasm. The values were not altered significantly even when the tumors had attained a very large size. Furthermore, this activity was independent of the strain, sex, and age of the animal.

The lowest values for the lactic and malic dehydrogenases were obtained in the dbrB adenocarcinoma and in the sarcoma 180. This would, perhaps, indicate a less efficient oxidative mechanism in tumor tissues. The value for the malic dehydrogenase, however, lies within the lowest range of those for normal tissues analyzed, viz., the spleen. This finding is in keeping with the observation of Greenstein (3) that the activity of the catalytic systems in tumors generally stands in the mid-region or near the lower extreme of the range of enzyme activities of normal tissues.

It is also significant that the enzyme preparations from the spleen of both normal and tumor-bearing animals failed in 72 per cent of the experiments to decolorize methylene blue completely when sodium lactate was the substrate. This phenomenon can probably be attributed to the low concentration of lactic dehydrogenase in this tissue, but the possibility of enzyme inhibitors cannot be excluded. A low concentration of enzyme systems requiring coenzyme I is indicated, however, since the addition of large quantities of coenzyme I failed to cause an appreciable acceleration of the rate at which aqueous extracts of spleen decolorized methylene blue (7). The fact that the values for the activity of malic dehydrogenase—another coenzyme I–requiring system—are lower for the spleen than for any of the other normal tissues analyzed further substantiates the supposition that certain enzymes requiring coenzyme I are present in small quantities in the spleen. Thus the failure of spleen extracts to decolorize methylene blue when lactate is the substrate can probably be ascribed to a low concentration of lactic dehydrogenase in this organ rather than to the presence of inhibitors of the enzyme system.

TABLE 1

Decolorization Time of Methylene Blue by Lactic and Malic Dehydrogenase in Skeletal Muscle of Normal and Tumor-bearing Mice

Strain of mice	Dehydrogenase	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}\times 10^2$)
			Normal animals—muscle			
dba	Lactic	14	5.8	(4.0–9.0)	±1.55	17.2
	Malic	18	4.7	(2.0–9.0)	±2.39	21.2
Rockland	Lactic	12	4.1	(3.25–5.0)	±1.65	24.4
	Malic	12	2.5	(1.0–8.0)	±2.50	40.0
C 57	Lactic	8	5.3	(4.5–9.0)	±0.65	18.8
	Malic	4	3.3	(3.0–3.5)	±0.24	30.3
			Tumor-bearing animals—muscle			
dbrB in dba	Lactic	20	6.5	(4.0–9.0)	±1.75	15.3
	Malic	30	4.5	(1.0–8.0)	±1.74	22.2
S 180 in Rockland	Lactic	14	4.8	(4.0–6.0)	±0.78	20.8
	Malic	14	2.6	(1.0–5.0)	±1.37	38.4

TABLE 2

Decolorization Time of Methylene Blue by Lactic and Malic Dehydrogenase in the Spleen of Normal and Tumor-bearing Mice

Strain of mice	Dehydrogenase	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}\times 10^2$)
			Normal animals—spleen			
dba	Lactic	16	>2 hours in 12 determinations; 60′ and 20′			
	Malic	14	11.0	(7–17)	±3.34	9.1
Rockland	Lactic	14	>2 hours in 10 determinations; 50′ and 30′			
	Malic	10	10.6	(7–18)	±3.83	9.4
C57	Lactic	8	>2 hours in 6 determinations; 22′			
	Malic	8	9.7	(4–12)	±3.34	10.3
			Tumor-bearing animals—spleen			
dbrB in dba	Lactic	18	>2 hours in 12 determinations; 30′, 22′, and 25′			
	Malic	22	9.5	(5–15)	±2.87	10.5
S 180 in Rockland	Lactic	10	>4 hours in 8 determinations; 30′			
	Malic	8	12.5	(11–14)	±1.12	8.0

TABLE 3

Decolorization Time of Methylene Blue by Lactic and Malic Dehydrogenase in Kidney of Normal and Tumor-bearing Mice

Strain of mice	Dehydrogenase	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}\times 10^2$)
			Normal animals—kidney			
dba	Lactic	22	4.0	(2.0–6.0)	±1.41	25.0
	Malic	26	2.2	(1.0–5.0)	±1.19	45.4
Rockland	Lactic	12	4.1	(3.0–6.0)	±1.34	24.4
	Malic	10	2.3	(1.5–3.5)	±0.68	43.5
C57	Lactic	12	4.0	(2.0–6.0)	±1.29	25.0
	Malic	10	2.6	(1.0–4.0)	±1.02	38.4
			Tumor-bearing animals—kidney			
dbrB in dba	Lactic	22	4.6	(2.0–7.0)	±1.91	21.7
	Malic	22	1.7	(1.0–3.0)	±0.81	58.8
S 180 in Rockland	Lactic	16	6.1	(3.0–10.0)	±2.03	16.4
	Malic	22	2.6	(1.0–6.0)	±1.48	38.4

TABLE 4

Decolorization Time of Methylene Blue by Lactic and Malic Dehydrogenase in Liver of Normal and Tumor-bearing Mice

Strain of mice	Dehydrogenase	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}$ × 10^2)
			Normal animals—liver			
dba	Lactic	18	4.9	(2.0–11.0)	±2.75	20.4
	Malic	28	2.4	(1.0–4.0)	±0.94	41.6
Rockland	Lactic	24	4.3	(3.0–10.0)	±1.99	23.2
	Malic	22	1.5	(1.0–4.0)	±0.92	66.6
C57	Lactic	20	5.6	(3.5–8.0)	±1.61	17.8
	Malic	10	2.4	(1.5–6.0)	±1.70	41.6
			Tumor-bearing animals—liver			
dbrB in dba	Lactic	32	5.8	(2.0–11.0)	±2.29	17.2
	Malic	30	2.2	(1.5–4.0)	±0.71	45.4
S 180 in Rockland	Lactic	16	6.5	(3.5–10.0)	±2.46	15.3
	Malic	30	2.6	(1.0–4.0)	±0.80	38.4

TABLE 5

Decolorization Time of Methylene Blue by Lactic and Malic Dehydrogenase in Brain of Normal and Tumor-bearing Mice

Strain of mice	Dehydrogenase	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Velocity (min.$^{-1}$ × 10^2)
			Normal animals—brain			
dba	Lactic	14	7.8	(3.0–13.0)	±3.13	12.8
	Malic	16	3.7	(2.0–8.0)	±1.86	27.0
Rockland	Lactic	14	7.3	(4.5–10.0)	±2.01	13.7
	Malic	16	3.5	(1.0–9.0)	±2.91	28.5
C57	Lactic	4	7.0	(6.0–8.0)	±1.00	14.3
	Malic	4	2.0	(2.0)	±0.00	50.0
			Tumor-bearing animals—brain			
dbrB in dba	Lactic	26	9.9	(5.0–14.0)	±2.87	10.1
	Malic	30	3.1	(1.0–7.0)	±1.74	32.3
S 180 in Rockland	Lactic	12	10.6	(6.0–16.0)	±3.99	9.4
	Malic	16	3.4	(2.0–6.0)	±1.64	29.4

TABLE 6

Decolorization Time of Methylene Blue by Lactic and Malic Dehydrogenase in Tumor Tissue

Strain of mice	Dehydrogenase	No. determinations	Decolorization time (min.) Mean	Range	S.D.	Veloc.ty (min.$^{-1}$ × 10^2)
dbrB in dba	Lactic	32	17.3	(12–22)	±3.38	5.7
	Malic	22	9.6	(5–12)	±2.22	10.4
S 180 in Rockland	Lactic	18	19.5	(15–24)	±2.79	5.1
	Malic	22	13.7	(7–20)	±4.31	7.3

SUMMARY

Studies of the comparative activities of lactic and malic dehydrogenases of the liver, kidney, brain, muscle, and spleen of various strains of mice revealed these enzyme activities to be independent of the strain, sex, and age of the animal. The presence of a transplanted tumor did not influence the activity of these enzymes in any of the organs analyzed. The values for lactic and malic dehydrogenases of a transplanted adenocarcinoma and sarcoma were lower than those of any non-neoplastic tissue studied, with the exception of the malic dehydrogenase of spleen from both normal and tumor-bearing mice. The malic dehydrogenase activity of the spleen corresponded closely to that of the same enzyme in both the dbrB adenocarcinoma and sarcoma 180. With lactate as substrate, enzyme preparations from the spleen of both normal and tumor-bearing mice failed to decolorize methylene blue completely in most cases. This can probably be attributed to the low concentration of lactic dehydrogenase in that organ.

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Serial Intraocular Transplantation of Frog Carcinoma for Fourteen Generations*

HANS SCHLUMBERGER, M.D.,† AND BALDUIN LUCKÉ, M.D.

(From the Laboratory of Pathology, School of Medicine, University of Pennsylvania Philadelphia 4, Pennsylvania)

The adenocarcinoma which commonly affects the kidneys of leopard frogs (*Rana pipiens*) may readily be transplanted to the anterior chamber of the eye (1, 2). Here the rate and manner of its growth can be observed directly through the thin, transparent cornea with the microscope, and permanent objective records can be obtained by photographs. Our previous studies have been based largely upon intraocular transplants during the first generation, i.e., transplants from primary tumors in the kidney. The present study deals with the behavior of tumors that became established in the eye and were then transplanted serially. The experiments are concerned with several interrelated questions: Is it possible to transplant the frog carcinoma for many generations and thus maintain it over a considerable period? If so, what effect will repeated transfer and prolonged maintenance in the anterior chamber have upon the tumor—upon its rate of growth; its manner of growth; its malignant properties, such as invasiveness; its response to changes in temperature; and its behavior when transplanted to alien species? Finally, how will a metastatic carcinoma behave in serial transplantation compared to a primary tumor?

MATERIALS AND METHODS

Details of the method for intraocular transplantation have been described in a previous publication (2). Briefly stated, smal portions of primary tumor, 1 to 2 mm. in size, were planted in the anterior chamber of the eyes of frogs with a finely pointed forceps through a small incision at the sclerocorneal junction. Aseptic precautions were observed, and care was taken to have the bits of tumor of approximately uniform size. The subsequent fate of the transplants was then observed at intervals of a few days through the slit-lamp microscope or a modification of the instrument. Permanent records were obtained by photographs.

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† Now at Ohio State University, Columbus, Ohio.

After the transplants had grown to fill the anterior chamber, the animals were anesthetized with ether, the cornea was cut away, the tumor removed and subdivided and reinoculated into the eyes of other frogs. The behavior of the new generation of tumors was then studied by the means outlined above.

In the main group of experiments the material for the first generation of transplants came from a large bilateral kidney tumor. The frogs used were unselected adults. They were kept separately in small aquarium jars in a moist atmosphere at a constant temperature of 28° C.

Details of the experiments as regards temperature, heterotransplantation, and transplantation of metastatic tumors will be given in the pertinent sections.

RESULTS

Incidence of takes and rate of growth in successive generations of transplanted tumors.—In the first generation the tumors grew in only 1 of the 10 frogs inoculated. When the tumor in this frog had filled the anterior chamber and bits of it had been retransplanted, 4 out of 5 transplants became established; in the next generation 6 out of 7 grew. After the third generation the incidence of takes rose to 100 per cent and was maintained at this level for the remainder of the experiment, i.e., from the fourth to the fourteenth generation (Table 1). The experiment was then discontinued, since it had shown that the kidney carcinoma of the frog can successfully be maintained in the anterior chamber for a considerable number of generations and over a period of 2 years and 3 months.

The form and size of all the tumors in successive generations were compared by means of photographs taken periodically, usually at weekly intervals. A representative group of such records is given in Figs. 1 to 6. The figures illustrate the pattern of growth which is characteristic of this tumor. In the early stages the transplants grew slowly; but, once they became established and vascularized, the rate was accelerated, and the

tumors enlarged rapidly. The final stage of growth is not shown in the photographs; as the mass filled the anterior chamber, increase in size became slower and slower.

The rate of vascularization and the size attained by the implants remained similar in all generations. Thus tumors in the fourteenth generation established vascular connections as quickly and attained as great a size as in earlier generations (Figs. 7 to 9).

The photographic records served as one means for comparing rates of growth in different generations. Another means for such comparison was the time required for the majority of transplants to fill the anterior chamber. Inspection of the last column in Table 1 shows that these times varied widely, from 37 to 100 days. There was no trend toward either a longer or a shorter rate in successive generations. Since, as stated above, care was taken to make transplantation as uniform a procedure as possible, the variations in growth rates must depend on unknown factors.

TABLE 1

SERIAL INTRAOCULAR TRANSPLANTATION OF FROG CARCINOMA THROUGH FOURTEEN GENERATIONS*

Generation of tumor	No. of animals inoculated	No. of takes	Average time for transplants to fill anterior chamber (days)
I	10	1	60
II	5	4	88
III	7	6	51
IV	10	10	38
V	7	7	48
VI	13	13	43
VII	14	14	60
VIII	11	11	46
IX	25	25	100
X	10	10	43
XI	23	23	79
XII	6	6	71
XIII	11	11	58
XIV	14	14	69
Total	166	..	854

* The table shows for each generation of transplanted tumors the number of frogs inoculated, the number of takes, and the time required for the majority of transplants to fill the anterior chamber. It is seen that the incidence of takes rose from 10 per cent in the first generation to a uniform 100 per cent after the third generation. The growth rates, however, did not become uniform but varied widely.

In confirmation of the view that serial transplantation did not effect a sustained change in rate of growth is a group of experiments which are summarized in Table 2. In several generations there were encountered transplants which had grown decidedly more rapidly or more slowly than their fellows. Thus, during the sixth generation in which the majority of the tumors required 43 days to fill the anterior chamber, one grew so rapidly as to require but approximately half this time, 22 days. But when transplants from this tumor were inoculated, the previous rapid rate was not maintained, the rate of growth approximating that of the controls, i.e., of the majority of tumors of the seventh generation (cf. first pair of figures in Table 2). Other and similar examples are given in the table, both for rapidly and for slowly growing tumors. Thus it may be concluded that no permanent alteration in rate of growth was developed by selective transplantation.

Manner of growth of transplants in successive generations.—Previous studies showed that the carcinoma when transplanted to the anterior chamber of the eye develops according to definite structural patterns, their type depending on the immediate physical environment (2). Three such morphogenetic patterns were recognized: (*a*) Where the tumor grew out into the aqueous humor, unimpeded by solid tissue, it formed cylinders, tubules, cysts, or papillary structures, which gradually lengthened and became more complex by branching. (*b*) Where the growing tips or margins of the outgrowths made contact with a firm surface, such as lens or cornea, adhesion took place and a change in form occurred; at this point the proliferating cells spread as flat membranes over the supporting surface. (*c*) Where contact was made with a loose, distensible tissue, such as iris, an invasive acinar type of growth resulted.

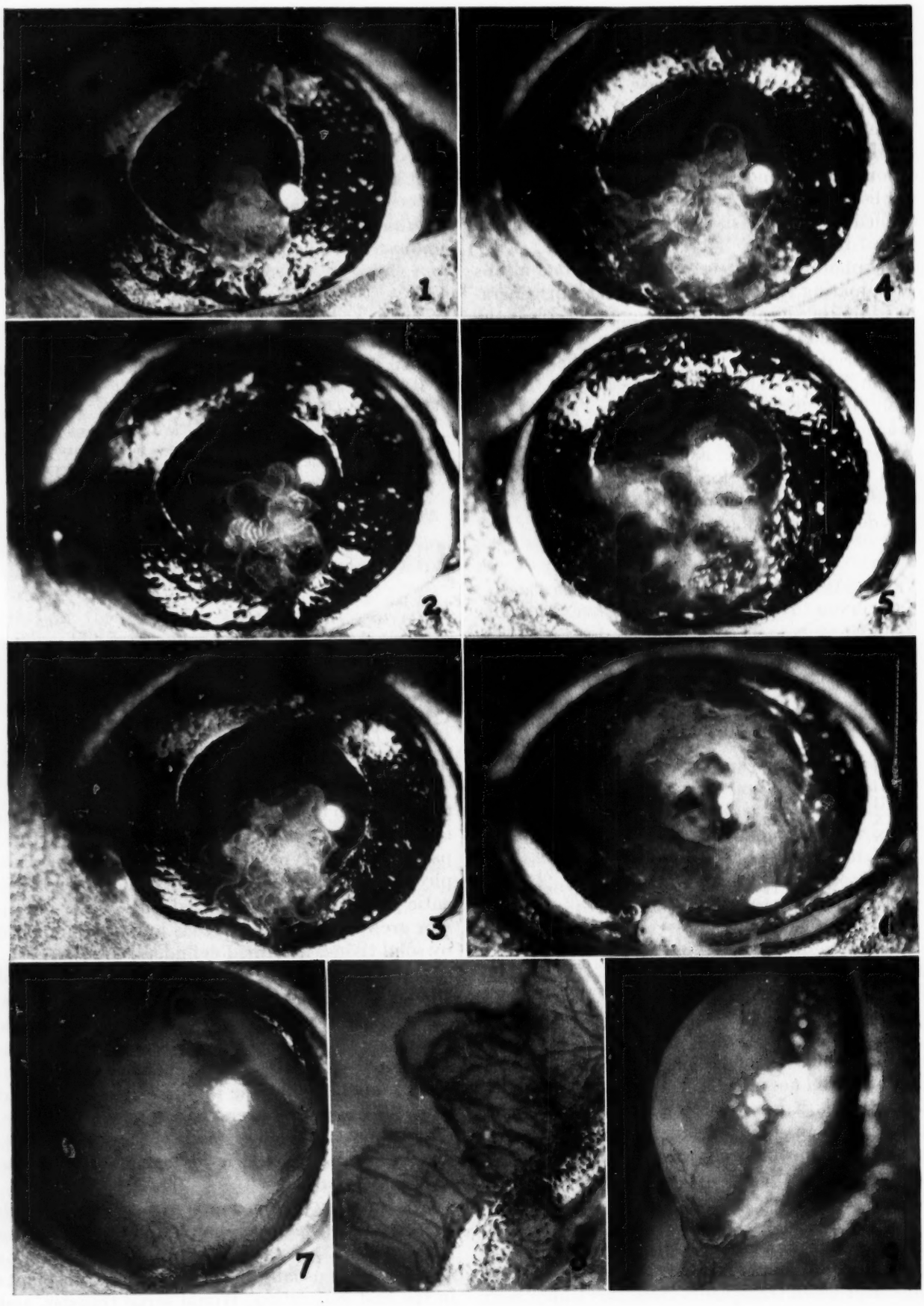
We have as yet encountered no factors which bring about a decisive change in this unvarying pattern. But, as stated above, our observations hitherto were usually made on tumor transplants in the first generation. Would serial transfer, and hence long residence in a new environment—the

TABLE 2

TIME REQUIRED TO FILL ANTERIOR CHAMBER UPON RETRANSPLANTATION OF STRAINS THAT PREVIOUSLY HAD DIFFERED FROM THE MAJORITY BY EITHER RAPID OR SLOW GROWTH

Designation of strain of tumor transplant	Time required by tumor to fill anterior chamber (days)	No. of animals subsequently inoculated	Average time for replanted tumors to fill anterior chamber (days)*
VI Rapid rate	22	6	57
VI Average rate	43	14	60
VII Rapid rate	38	6	64
VII Average rate	60	11	46
XI Rapid rate	47	9	71
XI Average rate	79	6	65
VII Slow rate	103	6	50
VII Average rate	60	11	46
VIII Slow rate	98	3	59
VIII Average rate	46	25	90

* Comparison of the pairs of figures in this column shows no significant differences in growth rates, i.e., no permanent alteration by selective transplantation has been effected.



FIGS. 1–9

aqueous humor—modify the growth pattern? The experiments demonstrated clearly that no changes in pattern developed. The tumors in all fourteen generations grew in a manner quite similar to that observed previously (3). Illustrative examples are to be seen in Figs. 1 to 6.

Development of corneal erosion.—We now come to a conspicuous effect of serial transplantation, i.e., increase in the ability of the tumor transplants to invade and destroy adjacent tissue. Although the frog carcinoma in its normal habitat, the kidney, is an invasive tumor which tends to destroy the surrounding renal tissue, its transplants to the eye, during the first few generations at least, very rarely invaded the ocular tissues other than the loose and spongy iris. The hard, unyielding cornea, on the contrary, usually offered an effective barrier to the spread of the cancer. Upon repeated transfer, however, and hence prolonged adaptation of the transplant to conditions prevailing in the eye, the tumor reacquired its invasive properties. Using perforations of the cornea as a readily visible criterion of invasion and subsequent destruction, it was found that such perforations occurred only irregularly and in relatively few animals during the earlier generations of transplants, whereas the incidence rose greatly in the later generations. Thus in the first seven generations of transplants, perforations of the cornea took place in only three, whereas in the following seven generations it occurred in all. The percentage incidence during the first seven generations was 12.6; it rose to nearly four times that rate, 44.8, during the following seven generations (Table 3).

The appearance and development of a corneal perforation is shown in Figs. 10 to 12. Fig. 10 illustrates a tubulomembranous growth in the eighth generation; it is adherent to the inner surface of the cornea, occludes the pupil, and has caused a slight outward bulging of the cornea. Careful examination with high magnification by the slit-lamp microscope, before and after instillation of fluorescein (an aid in making injuries to the outer surface visible), failed to reveal any abrasion of the corneal surface. During the following 17 days the lesion changed very little; but a second, larger, and complete perforation developed near by, through which portions of the tumor protruded (Fig. 11). During the following 3 weeks the eroded area rapidly increased in size; it had a punched-out appearance, and the protruding tumor gradually sloughed off (Fig. 12). In the example given, only one complete erosion occurred, even though another area of corneal bulging was present. Occasionally, two separate corneal erosions were encountered (Fig. 19).

TABLE 3

INCIDENCE OF CORNEAL PERFORATION IN SUCCESSIVE GENERATIONS OF INTERAOCULAR TRANSPLANTS OF FROG CARCINOMA*

Generation no.	No. of animals living over 21 days	No. of animals with corneal perforation	Percentage with corneal perforation
I	10	0	0
II	5	0	0
III	7	2	28.5
IV	7	2	28.5
V	5	0	0
VI	10	0	0
VII	14	3	21.4
VIII	6	1	16.6
IX	17	7	41.2
X	10	8	80.0
XI	23	10	43.5
XII	6	2	33.3
XIII	11	5	45.5
XIV	14	6	42.9

* Corneal perforation occurred irregularly and in relatively few animals during earlier generations of transplants. After the eighth generation the incidence rose greatly.

Once an erosion had developed, the tumor usually protruded through the opening. As a rule, the mass sloughed off, through injury or infection. But sometimes tumors that were well vascularized continued to grow for weeks, even though they were exposed; in such cases the nictitating membrane had served as a protecting envelope (Figs. 16 to 18).

FIGS. 1 to 9.—All figures are unretouched photographs of living tumors. All magnifications are at ×9, excepting Fig. 8, which is at ×20.

FIGS. 1 to 6.—Rate of growth of representative transplants in the eighth generation. The photographs were taken 12, 19, 26, 33, 40, and 51 days, respectively, after transplantation.

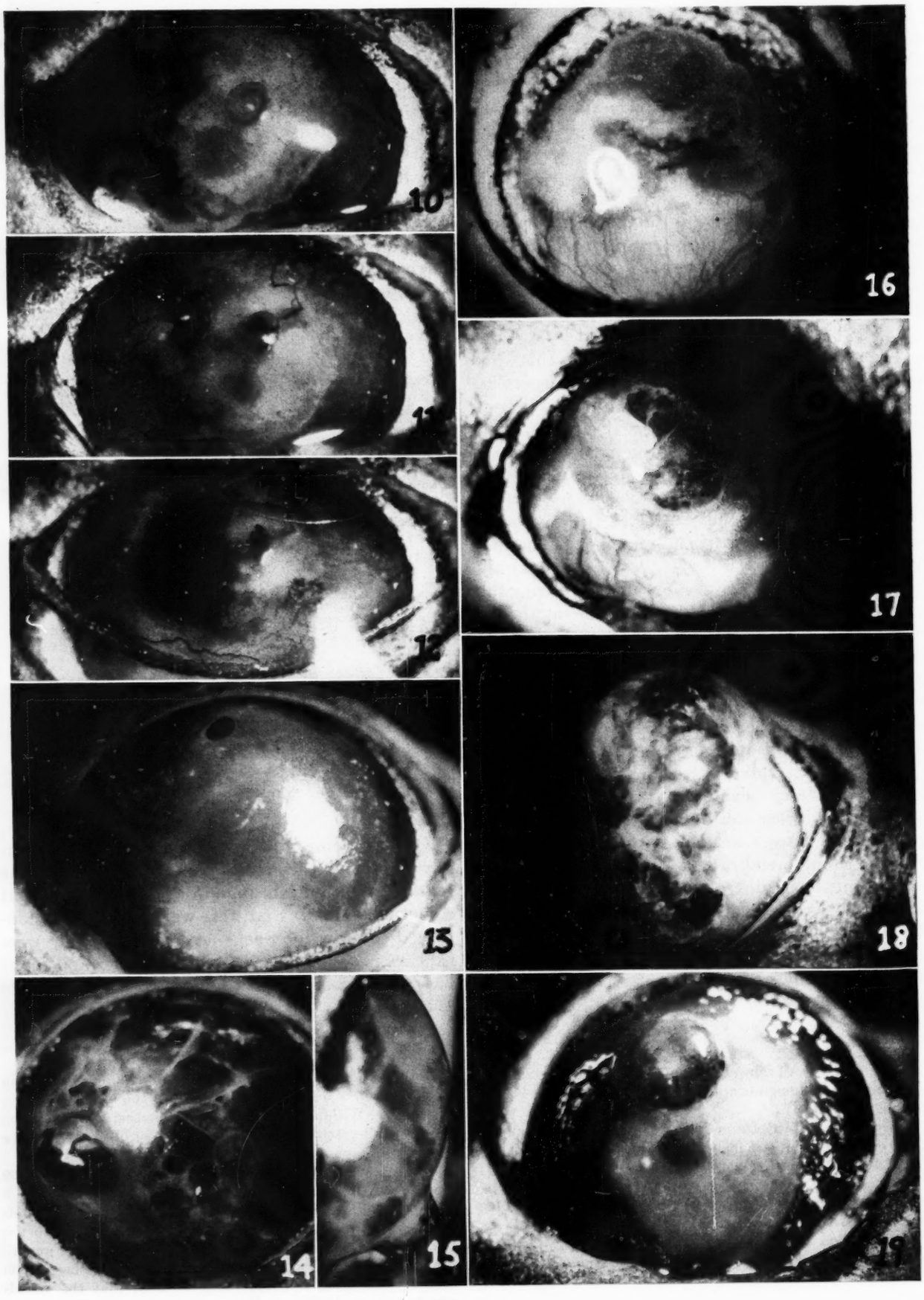
FIGS. 1 and 2.—During the earlier stages the outgrowths were tubules which rapidly became distended with fluid, thus forming small cysts.

FIGS. 3 and 4.—Through thickening of their walls and budding inward of new tubules, the cysts gradually became transformed into solid masses.

FIGS. 4 and 5.—Contact of the growth with lens resulted in the formation of a membranous carpet of tumor cells.

FIG. 6.—Within 11 days after last photograph was taken the now well-vascularized tumor had almost filled the anterior chamber.

FIGS. 7 to 9.—A tumor in the fourteenth generation, 69 days after transplantation. Fig. 7 gives a front view, Fig. 9 a side view, of the growth. In both photographs note the vessels which arise at the sclerocorneal junction and pass into the tumor. Fig. 8 is a photograph of these vessels at higher magnification. By careful focusing of the microscope and observation of the blood flow, it could easily be seen that the vessels penetrated into the tumor tissue.



FIGS. 10–19

As in the destruction of any tissue by a neoplasm, the mechanism of corneal erosion is complex and not completely understood. A constant factor is adhesion of an actively growing tumor to the inner surface of the cornea. A factor of importance, no doubt, is invasion of this structure by tumor cells which insinuate themselves into the interstices, thereby interfering with nutrition and weakening this normally tough tissue. Also, increased intraocular tension due to growth of tumor must play a part; it manifests itself especially in the areas of lessened resistance, where it leads to localized bulging (Figs. 14 and 15). Erosion, however, may sometimes develop without previous local bulging; in such cases the area to which the intraocular tumor is adherent rapidly becomes punched out as if the cells had undergone lysis (Fig. 13).

In brief, these experiments indicate that one of the malignant properties, i.e., invasiveness, of intraocular transplants of the frog carcinoma is enhanced by repeated transfer and prolonged maintenance in the anterior chamber of the eye.

The effect of temperature on growth of transplants.—The frog carcinoma is particularly good material for studying the effects of temperature on growth of neoplasms. Since the temperature of the frog is practically that of its surroundings, we have an opportunity to investigate the effects of temperature over a far wider range than is possible in warm-blooded animals. Previously we reported experiments in which the effect of temperature was studied by direct microscopic examination of living intraocular transplants mainly in the first generation, with a few, however, observed during four generations (3). It was found that at a high temperature, 28° C., the rate of growth was much accelerated, that the tumors became more rapidly vascularized, and that the tubular outgrowth tended to become cystic through accumulation of fluid. At a low temperature, 7° C., growth was greatly retarded but did not cease entirely; vascularization was poor, the outgrowth tended to be short and stubby, and rarely became cystic.

In our present experiments, all the tumor-bearing frogs were kept at a constant temperature of 28° C. It seemed of interest to find out whether prolonged sojourn at this abnormally high temperature had so altered the neoplastic tissue that it could no longer survive at a low temperature. Accordingly, when the tumors had been growing at 28° C. for 94 weeks and were in the twelfth generation, the frogs were placed in a constant-temperature room at 7°, where they were kept for 4 weeks. During this time the transplants remained practically unchanged in size; there was little or no evidence of growth. But the exposure to this low temperature had not harmed the tumors; for, when the frogs were returned to 28°, outgrowths became apparent within 3 days and continued without interruption in a manner entirely similar to the growth in the control group, which had remained at high temperature. A group of representative photographs are given in Figs. 20 to 23.

The experiments indicate that growth of intraocular transplants is not altered either quantitatively or qualitatively by long exposure to a high temperature.

The effect of serial transfer on subsequent heterotransplantation.—From earlier experiments we had learned that the kidney carcinoma of the leopard frog may as readily be established in the eyes of other species of the same family of frogs as in the natural host. In a species from a different family (the toad) the proportion of successful transplantations was somewhat less; but in animals of different classes of cold-blooded vertebrates the transplants regressed (4). It seemed quite possible that, after 2 years of growth in the anterior chamber, the tumor might be more adapted to the

FIGS. 10 to 19.—All figures are unretouched photographs of living tumors. Mag. ×9. *The photographs show the development and appearance of corneal perforations.*

FIGS. 10 to 12.—In Fig. 10 is shown a flat tubulomembranous growth from a transplant in the eighth generation. The tumor is adherent to the central portion of the cornea, which is bulging outward. Fig. 11 shows the appearance 17 days afterward; the bulge has now become eroded. A second, larger erosion has developed near by, through which parts of the tumor are protruding. By close inspection of the photographs it may be seen that numerous small vessels extend from the sclerocorneal junction to the edge of the larger erosion. Fig. 12 shows the eroded area as it appears 3 weeks after the preceding photograph was taken. The second erosion has become much larger. Note the abundant vessels supplying the tumor.

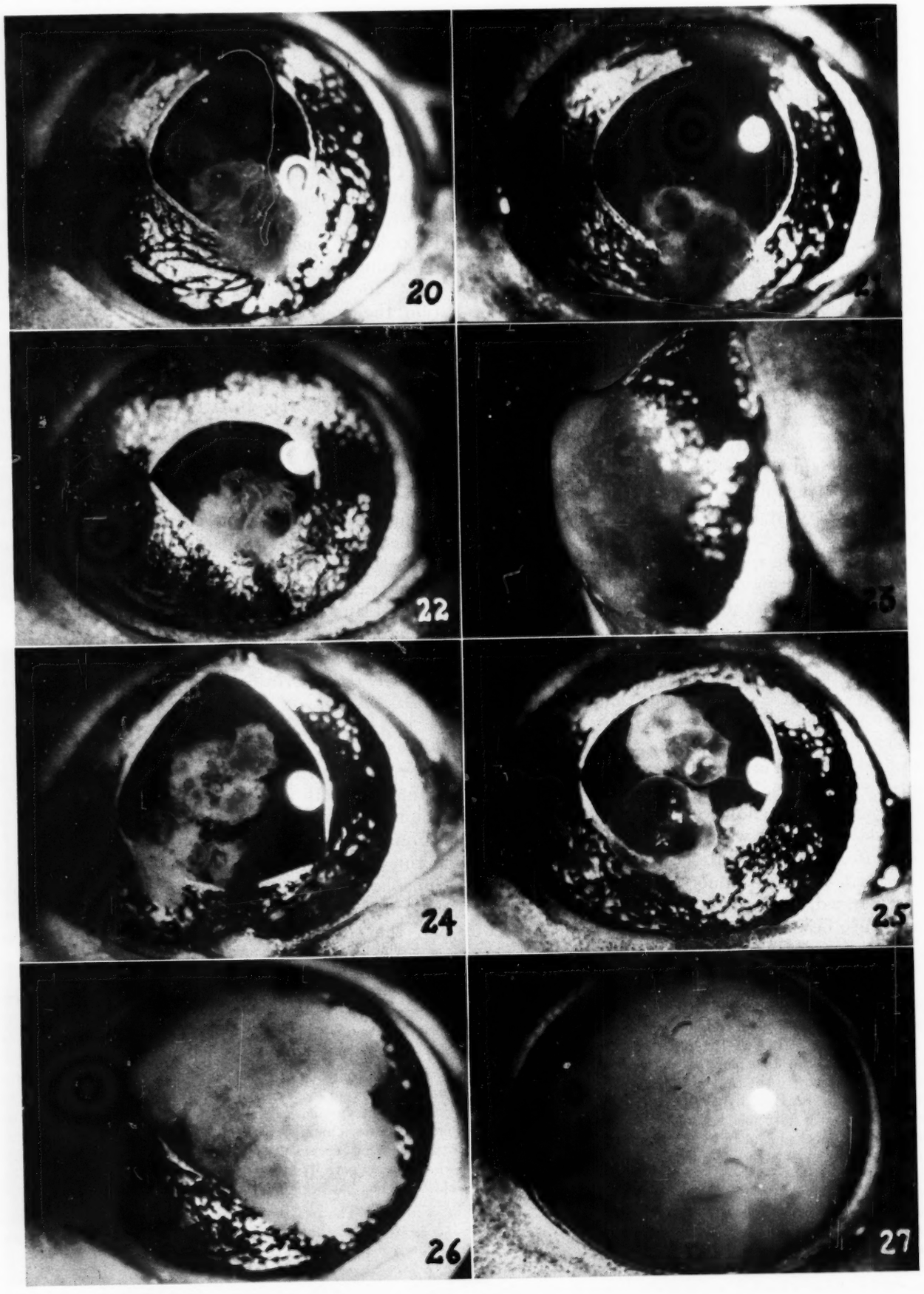
FIG. 13.—This photograph of a transplant in the twelfth generation shows a sharply circumscribed area of corneal destruction which is not accompanied by protrusion of the tumor.

FIGS. 14 and 15.—Front and side views of a corneal perforation through which the tumor protrudes. Note that the normal curvature of the cornea is retained except at the point of erosion. (From a transplant in the tenth generation.)

FIGS. 16 to 18.—In Fig. 16 is shown the appearance of a tumor in the tenth generation, 20 days after inoculation. Note the excellent vascularization. Eleven days later (Fig. 17) the entire anterior chamber is filled with tumor, and a large centrally located corneal perforation has developed, through which the mass protrudes. During the next 11 days almost the entire cornea has been destroyed.

FIG. 18.—The protruding tumor has been protected from injury by the nictitating membrane (which in the photograph has been pulled away).

FIG. 19.—This tumor in the seventh generation occupies only a portion of the anterior chamber, but two perforations have developed.



FIGS. 20–27

aqueous humor of alien species. However, this did not prove to be the case. When in the thirteenth generation the neoplasm was transferred to the eyes of 4 bullfrogs (*Rana catesbiana*), 6 toads (*Bufo americanus*), and 3 goldfish (*Carassius auratus*), it grew in all the bullfrogs (causing erosion of the eye in 1); in the toads it became quickly established in 2 and grew more slowly in 2 others. In the goldfish the transplants were promptly surrounded by a cellular exudate and at no time showed growth. Thus, while serial transplantation had increased the number of takes in the natural host, it had not facilitated transplantation to alien hosts.

Serial transplantation of a metastatic tumor.—The formation of metastatic colonies by a cancer indicates that its malignant properties are fully developed; for its cells are now able to grow in tissues different from those of their origin (5). How would metastatic tumors behave when serially transplanted? Would they grow more luxuriantly in the anterior chamber of the eye than did primary tumors? The material for investigating these questions was a large kidney tumor which had formed numerous colonies in the liver. Some of these were transplanted into the anterior chamber of 9 frogs, each animal receiving an entire small metastatic nodule. Nine other frogs were similarly inoculated with bits of the primary tumor The subsequent experimental procedures were identical with those described in the introduction. None of the transplants of the primary tumor became established and all soon regressed. On the contrary, 1 of the 9 metastatic nodules grew vigorously; upon retransplanting it to the second generation, 10 of 11 tumors became established; thereafter, all the transplants grew (Table 4). This incidence of takes is very similar to that obtained for the primary tumor, studies of which have been summarized in Table 1. The transplants of the metastatic tumors were maintained for 385 days, during which they were passed through five generations. Their pattern of growth was entirely similar to that of transplants from primary tumors. There was, during early stages, a period of relatively slow growth, followed by a later period of rapid growth (Figs. 24 to 27). The characteristic formations of tubules, cysts, and membranes were all observed. The rate of growth tended to be somewhat slower than the rate reported in Table 1 for a primary tumor, but no significance can be attributed to such minor variations.

The invasiveness of the metastatic tumor was not greater than that of a primary neoplasm when transplanted to the eye. Corneal invasion and perforation occurred in only 2 of the 43 animals; this is in keeping with the low incidence during earlier generations of implants from primary tumors. The experiments were discontinued after the fifth generation because it was evident that transplants from metastatic tumors reacted in the same way as did primary tumors.

TABLE 4

SERIAL INTRAOCULAR TRANSPLANTATION OF METASTATIC CARCINOMA THROUGH FIVE GENERATIONS*

Generation of tumor	No. of frogs inoculated	No. of takes	Average time for transplants to fill anterior chamber (days)
I	9	1	83
II	11	10	88
III	7	7	70
IV	7	7	74
V	9	9	70

* Except for a somewhat slower rate of growth, the metastatic carcinomas behave similarly to the primary tumor, studies of which are summarized in Table 1.

SUMMARY

A renal carcinoma of the leopard frog has been successfully maintained in the anterior chamber of the eye through fourteen serial transplantations over a period of 2 years and 3 months. The characteristics of the growth of the tumor were observed *in vivo* with the slit-lamp microscope. After

FIGS. 20 to 27.—All figures are unretouched photographs of living tumor. Mag. ×9.

FIGS. 20 and 21.—The effect of low temperature on growth of tumor. Fig. 20 shows the appearance of a transplant in the twelfth generation on the day that the frog, previously kept in a thermostat room at 28° C., was transferred to a constant temperature of 7° C. After remaining at the low temperature for 4 weeks, the tumor is seen to be almost unchanged; there is very little evidence of growth (Fig. 21).

FIGS. 22 and 23.—The effect of high temperature on tumor growth. These photographs are of transplants belonging to the same series as those of the two preceding figures. The animal, which had been left at 28° C., remained at this temperature for 4 additional weeks. In Fig. 22 is given the appearance of the transplant at the beginning of the experiment; in Fig. 23 at the end, the anterior chamber has almost been filled by the vigorous growth. Figs. 22 and 23 should be compared with Figs. 20 and 21, respectively, to show the growth-accelerating effect of temperature.

FIGS. 24 to 27.—Growth of a liver metastasis after five serial transplantations in the eye. Fig. 24 shows the appearance of the tumor 1 week after inoculation; Fig. 25, the appearance 1 week later. In Fig. 26, taken 11 weeks after inoculation, about two-thirds of the anterior chamber has become filled; 1 week afterward the entire chamber has been occupied (Fig. 27). Compare the relatively slow rate during the first 2 weeks of the experiment with the rapid rate of growth during the last week.

repeated transfer, the growth of the implants became more invasive, as indicated by increasing frequency of corneal erosion. The general pattern of growth, however, as well as the response to temperature and heterotransplantation remained remarkably stable.

Metastatic tumors located in the liver were similarly studied in five generations. Both in vigor and in pattern the growth of these transplants was found to correspond closely to that of primary tumors. These results suggest that primary tumors and their metastases will grow in like manner when placed in a similar environment.

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Transplantation of Heterologous Tumors by the Intravenous Inoculation of the Chick Embryo*†‡

DORIS H. BENDER, M.D., CHARLES E. FRIEDGOOD, M.D., AND HENRY F. LEE, M.D.

(From the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania and the Children's Hospital of Philadelphia)

This report is concerned with the transplantation of cells of certain mammalian neoplasms into the living chick embryo by injection of these cells into the allantoic vein. Although the technic of allantoic vein injection has been reported previously, it has not been used heretofore as a technic for implanting tumor cells from the same species or from another.

It is well known that tissues transplanted from one species to another fail to grow except under certain conditions. Such heterologous transplants have been successful in the anterior chamber of the eye and on the chorio-allantois or in the yolk sac of the chick embryo. In specific instances other methods have had limited success.

The fact that many types of tissue have been grown for short periods of time on the chick chorio-allantois may be related to the absence of antibody formation. The absence of complement from the serum of the chick embryo was noted as early as 1907 (1). In 1929, Grasset (2) observed that the chick embryo lacked the ability to produce antibody. Polk, Buddingh, and Goodpasture (3) in 1938 showed that complement for sensitized sheep cells was not present in the serum, extra-embryonic fluids, or tissues of the chick embryo before hatching. Just at hatching or immediately afterward it was suddenly present and gradually increased to a maximum in the adult fowl. Murphy (4) in 1914 observed that there was no reaction upon the part of the chick embryo to foreign tissue growing on the chorio-allantois until the eighteenth day of incubation.

The failure of growth of heterologous tissue, either normal or neoplastic, has been thought to be based on immunity reaction and species specificity. Therefore, it seemed reasonable that successful transfer of heterologous tumor tissue might be effected in the chick embryo if only for a limited period of time. The presence of growth-promoting substances in the rapidly growing chick embryo should make it an ideal medium for growth of heterologous tissue. Furthermore, it seemed that the environment of the tumor tissue within the embryo itself would approximate more closely the environment of such tissue as seen in patients than would tumor growth upon the chorio-allantois or within the yolk sac.

Previous experience of Lee, Stavitsky, and Lee (5) and Lee and Stavitsky (6) with intravenous inoculation of chick embryos with suspensions of *Mycobacterium tuberculosis* suggested that this method might be useful in establishing heterologous tumor growth within the embryo. The use of this technic also offered an opportunity to determine what tissues were most likely to be the site of metastasis following blood stream dissemination.

Sterile tumor tissue was obtained from patients at operation, from tumor-bearing rats and mice, and from tissue culture. The tumor tissue was collected under aseptic precautions. It was then forced through a 70-mesh Monel wire screen, and a sterile suspension of tumor cells in physiological saline was prepared. Lewis (7) has determined that such suspensions contain viable cells.

Chick embryos incubated for 11 days were selected and the eggs candled. A portion of the egg shell over the air sac was removed and the shell membrane carefully stripped off, exposing the chorio-allantois. An inoculum of 0.05 cc. of the tumor-cell suspension was then injected intravenously. The eggs were sealed with Scotch tape, which formed a window through which one could observe the developing chick embryo. The surviving embryos were sacrificed on the twentieth day of incubation, or 9 days after inoculation.

In all, 278 embryos were injected intravenously with 0.05 cc. of sterile tumor-cell suspension in a series of 17 experiments. Following the intravenous injection of physiological saline alone in 11-day-old

* Read at the annual meeting of the American Association for Cancer Research, Atlantic City, N.J., March, 1948.

† This work was carried out in the laboratories of the Henry Phipps Institute, of the University of Pennsylvania.

‡ The equipment was supplied by the Heyden Chemical Corporation.

embryos, 70 per cent survival is expected. Survival following the intravenous inoculation of chick embryos with sterile tumor-cell suspensions averaged about 50 per cent. This survival has been improved recently by the addition of small amounts of penicillin to the tumor-cell suspension, which is allowed to stand at room temperature for ½ hour prior to inoculation into the embryos.

By this technic the C57 strain mouse sarcoma (Table 1) was successfully grown. In 81.7 per cent of surviving embryos injected with this tumor-cell suspension no growth was demonstrated, while 18.3 per cent of surviving embryos showed evidence of tumor growth. In several cases the growth was small, but definite nests of tumor cells could be found histologically in sections of the liver or brain or both (Figs. 1–3). No evidence of tumor metastasis was found in the kidney or spleen. In one instance, although the chick itself appeared to be normal in size and development, diffuse nodulation of the liver was observed grossly. On histologic examination, about one-fourth of the liver tissue appeared to be replaced by tumor tissue, and the brain was diffusely invaded by nests of tumor cells. These appeared to be similar to the parent tumor cells. Mitoses were present as was frequent blood vessel invasion. Occasionally a bile canaliculus appeared to be invaded by tumor cells.

TABLE 1

THE GROWTH OF RODENT TUMORS IN THE CHICK EMBRYO

Tumor	Time interval between removal of tumor from source and injection into chick	No. of embryos surviving after intravenous inoculation	No. of takes
C57 strain mouse sarcoma	1 hour or less	60	11 (18.3%)
C_3H strain mouse carcinoma	1 hour or less	18	0
Total		78	11 (14.1%)

The C_3H strain mouse mammary carcinoma failed to grow.

Tumor transfer from human beings (Table 2) was accomplished in four instances. Nine and six-tenths per cent of the surviving embryos injected with such tumor suspensions showed microscopic takes in sections of the liver but not in other embryonic tissues. These takes were all small. The tumors from patients which showed evidence of growth within the embryo were two neuroblastomas, a cerebral hemangioblastoma, and a cerebral metastasis secondary to a bronchogenic carcinoma. The lapse of time between removal of the tumor from the patient and inoculation of the tumor-cell suspension into the chick embryo may be responsible for the few takes and small growth of human tumor tissue within the embryo.

Following the intravenous injection of normal cells from the embryonic chick liver or brain, perivascular cellular infiltration and occasional focal necroses were seen histologically in the livers of the surviving embryos, but there were no changes suggestive of tumor formation.

TABLE 2

THE GROWTH OF HUMAN TUMORS IN THE CHICK EMBRYO

Tumor	Time interval between removal of tumor from source and injection into chick (hours)	No. of embryos surviving after inoculation	No. of takes
Neuroblastoma	6	9	1
Chondrosarcoma (cadaver)	4	2	0
Metastatic adenocarcinoma*	30	0	0
Medulloblastoma	7	4	0
Neuroblastoma	7	4	1
Neurofibroma (cerebral)	7	5	0
Hemangioblastoma (cerebral)	2	3	1
Metastatic carcinoma† (cerebral)	3	15	1
Metastatic adenocarcinoma‡	10	1	0
Total	..	43	4 (9.6%)

* Probably from colon.
† Probably from lung.
‡ Probably from cecum.

TABLE 3

SUMMARY

No. of chicks injected I.V. with tumor-cell suspensions	No. of survivors	No. of takes
278	121	15 (12.4% of survivors)

In the entire series of experiments there was evidence of tumor growth in the brain or liver of 12.4 per cent of surviving embryos injected intravenously with tumor-cell suspensions. The neoplastic cells maintained their histologic character in the embryo and closely resembled the parent tumors. This morphologic evidence suggests the probable identity of the transplants with the parent tumors. Experiments are now under way to effect serial transfer of heterologous tumor transplants. In the case of transplants of animal tumors we are attempting to return such transplants to a member of the original host species. Attempts are

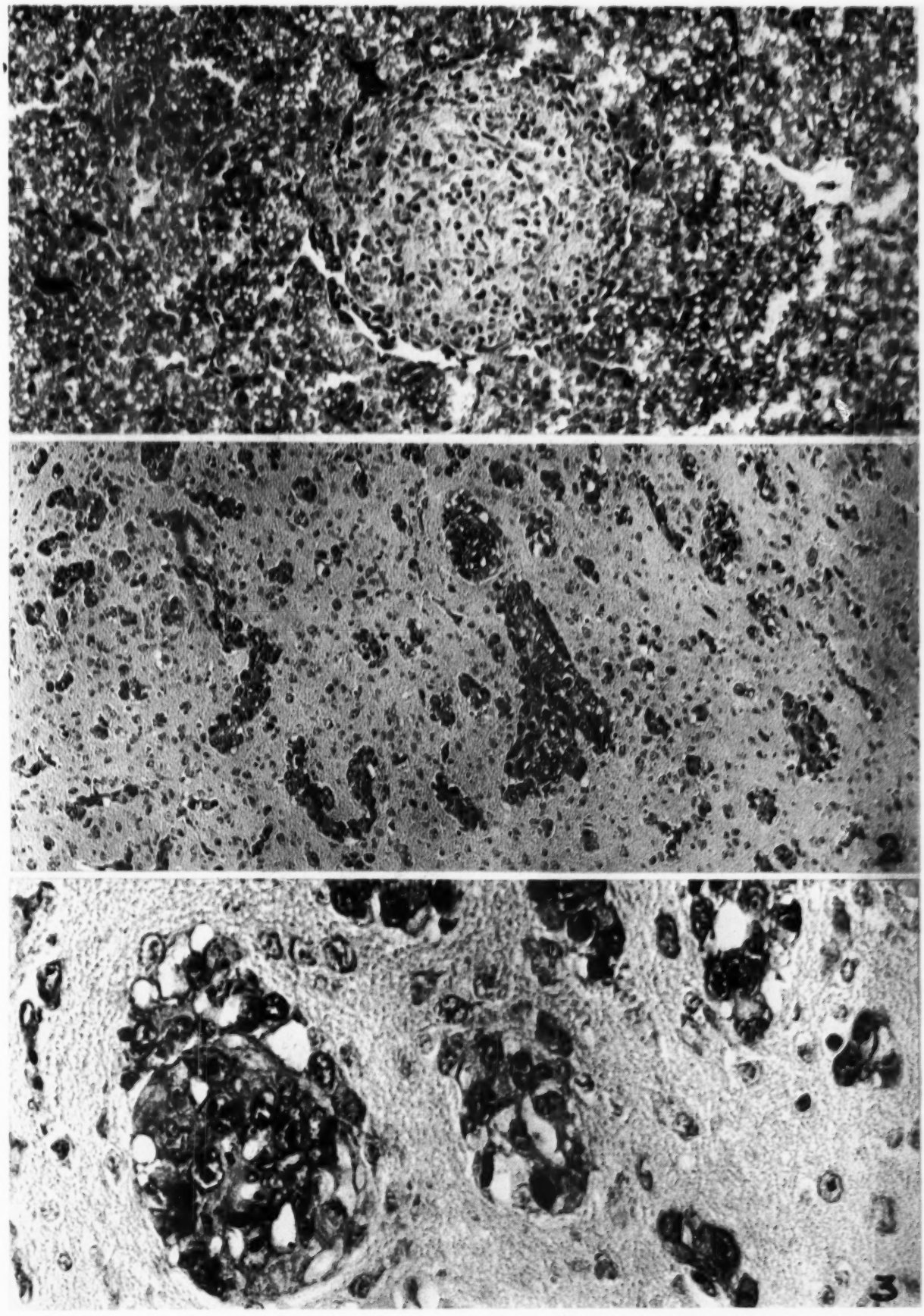

FIG. 1.—Metastatic nodule in the liver of a 20-day-old chick embryo following intravenous inoculation with the C57 strain mouse sarcoma. Mag. ×330.

FIG. 2.—Diffuse involvement of the brain of a 20-day-old chick embryo injected intravenously with the C57 strain mouse sarcoma. Mag. ×160.

FIG. 3.—Higher magnification of a section of the brain of a 20-day-old chick embryo injected intravenously with the C57 strain mouse sarcoma. Mag. ×660.

also being made to determine what happens to the tumor transplants after the chick is allowed to hatch.

At this time it can be stated that it is possible to grow heterologous tumors by the intravenous inoculation of the chick embryo with sterile tumor-cell suspensions, although we have not been able as yet to propagate tumors by this method consistently.

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